Thrombotic tendency in young stroke patients : the thrombin potential as a screening parameter for coagulation disturbances

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Thrombotic tendency in young stroke patients: the thrombin potential as a screening parameter for coagulation disturbances
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Thrombotic tendency in young stroke patients

The thrombin potential as a screening parameter for coagulation disturbances

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People do not lack strength; they lack will

(Victor Hugo)

voor Henk en Thomas

Contents

Abbreviations

APC	activated protein C
ESR	erythrocyte sedimentation rate
ETP	endogenous thrombin potential
GP	glycoprotein
INR	International Standardized Ratio
OR	odds ratio
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PPP	platelet poor plasma
PPA	platelet derived procoagulant activity
PRP	platelet rich plasma
PS	phosphatidyl serine
RBCA	red blood cell aggregation
TF	tissue factor
TIA	transient ischaemic attack
TM	thrombomodulin
vWF	von Willebrand factor

General introduction

Stroke is the third leading cause of death and an important cause of long-term disability in most industrialised populations.[1] Stroke incidence rates strongly rise with age. Up to 10% of stroke patients is younger than 45 years.[2] Especially stroke in young persons has dramatic consequences because permanent sequelae of stroke may remain for another 30 to 50 years. Moreover, it heavily burdens not only the patient, but also the partner, family, and society in general. Approximately 60% of strokes in young patients are infarctions.[3,4]

The causes of ischaemic stroke in the young are diverse and consequently require a thorough diagnostic workup. Despite extensive investigations the cause of stroke remains uncertain in many young patients. This thesis is concerned with those causes that can be attributed to changes in haemorheology and haemostasis. In healthy persons a delicate balance exists between the coagulant and the anticoagulant system. This balance assures the fluidity of blood as well as adequate haemostasis. Stroke is one of the most dramatic signs of a disequilibrium of the coagulation system. Haemorheologic factors, like red blood cell aggregation and blood viscosity, also influence blood flow. In 1990 a haematologic disorder or coagulopathy could be identified as the likely cause of stroke in only approximately 4% of young patients.[5] Whether a coagulopathy is identified as a possible contributor to the development of stroke depends on the availability of valid tests that detect such disorders.

An enhanced erythrocyte aggregation is one of the possible causes of stroke.[6] Enhanced red blood cell aggregation was correlated to fibrinogen plasma level, which in itself is a risk factor for stroke. In chapter 2 we tested (1) whether erythrocyte aggregation is indeed enhanced in young stroke patients and (2) to what extent enhanced red blood cell aggregation is linked to fibrinogen.

The putative role of abnormalities of the clotting system suggests that screening for coagulation defects in young stroke patients may be useful. Until recently a sensitive test of the overall haemostatic function of blood has been lacking because clotting times are not sensitive to hypercoagulability. The clotting time does not reflect the amount of thrombin that will be formed, because clotting occurs as soon as 5-10 nM of thrombin are formed, whereas thrombin generation continues until a peak of about 200 nM is reached. Therefore, we have used a new assay, the thrombin potential, i.e. the area under the thrombin generation curve. The thrombin potential gives information on the coagulation system also in case of hypercoagulability and

hence could be a useful screening parameter. With the use of the thrombin potential, patients with coagulation abnormalities could be identified, thereby limiting expensive further laboratory investigations to patients with an identified abnormality of overall function of the clotting system. To detect abnormalities in the plasmatic coagulation system we investigated the thrombin potential in platelet poor plasma. As mentioned above the haemostatic system is an equilibrium between the coagulant and the anticoagulant pathways. Therefore, we also investigated the role of the protein C pathway. We performed this by measurement of the thrombin potential in platelet poor plasma in the presence of thrombomodulin, which together with thrombin activates protein C. Activated protein C can inhibit activation of factor V and factor VIII (chapter 3).

Apart from the plasmatic coagulation system, also platelets and von Willebrand factor are essential in thrombogenesis, among others via their role in thrombin formation.[7] Comparing the thrombin potential in platelet rich plasma to that in platelet poor plasma allows distinction between hypercoagulability due to platelet abnormalities and plasma based hypercoagulability. Therefore, we investigated the thrombin potential in platelet rich plasma in young stroke patients and in controls. We Also measured the platelet derived procoagulant activity in serum, resulting from the activated platelet rich plasma. Recently, von Willebrand factor appeared a risk factor in the development of stroke.[8,9] We therefore also measured the level of von Willebrand factor (chapter 4).

Up till now, most patients with ischaemic stroke of presumed arterial origin receive antiplatelet therapy (aspirin) as a means of secondary prevention. However, the benefits are moderate. Aspirin administration in patients with a prior stroke or transient ischaemic attack results in an absolute risk reduction in non-fatal stroke of 2% (8.2% in treated patients, 10.2% in controls), i.e. a relative risk reduction of 20%.[10] The alternative is oral anticoagulation. High-dose anticoagulants carry a substantial risk of adverse events, especially cerebral bleeding complications.[11] Therefore, high-dose anticoagulants are not indicated in the prevention of non-cardioembolic stroke. Treatment with fixed low-dose oral anticoagulants lowered the risk of thromboembolic complications in some studies, but not in all.[12-15] The use of oral anticoagulants at very low intensity might be an alternative in patients requiring long-term thrombosis prophylaxis. Such a regimen, if efficacious, might be associated with a lower bleeding risk than warfarin at standard intensity. It also might require less frequent laboratory monitoring. The thrombin potential is known to decrease with full oral anticoagulant therapy, but the effect of low-dose aspirin and fixed low-dose anticoagulants is not known. Hence the reason why we investigated the influence of antiplatelet therapy (aspirin) and fixed low-dose anticoagulants (phenprocoumon 0.75

mg daily) on thrombin generation both in platelet rich plasma and in platelet poor plasma in young stroke patients and in healthy subjects (chapter 5).

In summary:

This thesis aims to answer the following questions:

1. Do young stroke patients have an enhanced red blood cell aggregation compared to elderly stroke patients and to controls? (chapter 2)

2. Is red blood cell aggregation a risk factor for stroke independent of the fibrinogen concentration? (chapter 2)

3. Could we, by measuring the thrombin potential in platelet poor plasma, identify a subgroup of young stroke patients with hypercoagulability due to the plasmatic coagulation system? (chapter 3)

4. Could we, by measuring the thrombin potential in platelet rich plasma, identify a subgroup of young stroke patients with hypercoagulability? (chapter 4)

5. Could we, by comparing thrombin generation in platelet rich plasma and in platelet poor plasma, identify a subgroup of patients with hypercoagulability due to platelet abnormalities? (chapter 4)

6. Do aspirin and fixed low-dose oral anticoagulants decrease thrombin generation in platelet rich plasma and/or in platelet poor plasma in young stroke patients and in healthy subjects? (chapter 5)

Chapter 

Ischaemic stroke in young patients
erythrocyte aggregation
platelet procoagulant activity
and thrombin generation

an introduction to this thesis

Ischaemic stroke in young patients

In the hospital-based primary stroke centres of Lausanne and Maastricht, 12% and 8% of all patients with an ischaemic stroke were younger than 45 and 50 years, respectively.[2 16] Of these young patients about two thirds presented with an infarct and one third with a transient ischaemic attack (TIA). Minor stroke and TIA patients do not differ in age, sex, vascular risk factors and underlying pathophysiology, whereas they have similar risks of future stroke or vascular death.[17] Therefore, they can be considered together for the use of stroke research and clinical trials.

The causes of ischaemic stroke in young patients are more diverse than in elderly patients. The most common causes of ischaemic stroke in the young are listed in table 1.1.[18] Ischaemic stroke can be categorized by location, infarct size, clinical features and underlying stroke mechanism. Lacunar infarcts are attributed to small-vessel disease with local obstruction of a small perforating artery deep in the brain or brainstem, whereas large-vessel disease causes larger, often called territorial infarcts. In general, unusual causes of stroke are more frequent among young patients than among the elderly, in whom atherosclerosis is by far the most frequent cause of ischaemic stroke. The prevalence of atherosclerosis increases with age. Overall, atherosclerosis has been considered to be the cause of stroke in 7 to 38% of patients younger than 50 years. Other frequent causes are arterial dissection and cardiogenic emboli. Despite extensive investigations, the cause of stroke remains uncertain in 7 to 56% of patients.[2 16 18-22] Unusual causes of stroke are more frequent in young patients with territorial stroke than in lacunar stroke.[23]

Until now, in up to a quarter of young patients with stroke, the major precipitant of brain ischaemia is a haematologic disorder or coagulopathy.[5 24 25] In elderly patients atherosclerosis is the most frequent cause of stroke. Prethrombotic states may contribute to stroke in elderly patients as well. The coexistent atherosclerosis in these patients could obscure the role of such a coagulopathy. A haematologic disorder, when identified after stroke, is not necessarily the cause of stroke, as it may be just a consequence. An antecedent haematologic disorder as a cause for stroke is more likely if it persists in the subsequent months following the ischaemic event. The state of the art in haematological research is such that many isolated causes of hypercoagulability can be demonstrated. Specific tests for these disorders are expensive and not readily available. Furthermore, they usually yield information only on a very small part of the coagulation system. Therefore, their sensitivity is rather low, which does not make them ideal to 'screen' for coagulation disorders in young stroke patients.

Table 1.1. Causes of ischaemic stroke in patients less than 50 years of age

1. Atherosclerosis	7-38%
2. Embolism	10-30%
a. Cardiac source	
- valvular disease	
- atrial fibrillation and sick sinus syndrome	
- acute myocardial infarction and/or left ventricular aneurysm	
- left atrial myxoma	
- cardiomyopathy	
b. Paradoxical embolism and pulmonary source	
- pulmonary arteriovenous malformation	
- atrial and ventricular septal defects with shunt	
- patent foramen ovale with shunt	
- pulmonary vein thrombosis	
- pulmonary and mediastinal tumors	
c. Other	
- aortic cholesterol embolism	
- transient embologenic aortitis	
- emboli distal to unruptured aneurysm	
- fat embolism syndrome	
3. Arteropathy	10-20%
a. Inflammatory	
- Takayasu's disease	
- allergic and granulomatous	
- infectious	
- associated with systemic disease	
b. Noninflammatory	
- spontaneous dissections	
- fibromuscular dysplasia	
- neck trauma	
- Moya moya syndrome	
- Familial: homocysteinuria, Fabry's pseudoxanthoma elasticum	
4. Haematologic diseases and coagulopathies	1-25%
a. Hyperviscosity	
- polycythemia and myeloproliferative disease	
- dysproteinaemia	
b. Coagulopathy	
- thrombotic thrombocytopenic purpura	
- diffuse intravascular coagulation	
- paroxysmal nocturnal hemoglobulinuria	
- oral contraceptive use/ peripartum/ pregnancy	
- thrombocythaemia	
- sickle cell disease	
- hyperhomocysteinaemia?	
- lupus anticoagulant?	
- protein C/S deficiency?	
- APC-resistance?	
- antithrombin III deficiency?	
- platelet hyperaggregability?	
5. Small-vessel disease	2-8%
6. Possibly	
a. Migraine	
b. Alcohol intoxication	
7. Undetermined	7-56%

There are a number of haematologic abnormalities, that may be related to ischaemic stroke in the young.

Deficiencies of coagulation inhibitors (antithrombin, protein C, protein S and heparin cofactor II) could theoretically increase arterial thrombosis. Yet, no convincing evidence is found for involvement of antithrombin-III deficiency, of protein C deficiency, of protein S deficiency, and of activated protein C resistance (factor V Leiden) in the development of arterial thrombosis.[5 24 26-30] However, recent data showed that young women with factor V Leiden mutation have an increased risk of myocardial infarction, but only when other risk factors are present (especially smoking).[31 32]

Lupus anticoagulant and anticardiolipin antibodies are acquired immunoglobulins that are associated with thrombosis. These are closely related autoantibodies that react with proteins associated with phospholipid. Reported frequencies of antiphopholipid antibodies in patients with stroke vary widely, with a prevalence of 1 to 50%.[24 33-38] A recent study in an unselected stroke population found no evidence to support the hypothesis that anticardiolipin antibodies are an independent risk factor for stroke in young people.[39] There was an increase in IgG titre with age and with the number of vascular risk factors, but the authors interpreted this as suggesting that anticardiolipin antibodies may be a nonspecific accompaniment of vascular disease. The overall contribution of antiphospholipid antibodies to the development of stroke therefore remains uncertain, but is probably very low.

Oral contraceptives and pregnancy are considered another cerebrovascular risk. Data on oral contraceptives are conflicting. Some authors did not find the overall risk of stroke to be increased among current users of low dose oestrogen oral contraceptives,[40] whereas others described a small increase in relative risk of occlusive stroke for women of reproductive age who currently use oral contraceptives.[41] The attributable risk, however, was very small. The high dose oestrogen pills carried a higher risk than the low dose pills, irrespective of the type of progestin. The risk of cerebral infarction was increased in the six weeks after delivery (adjusted relative risk 8.7) but not during pregnancy itself.[42]

Hyperhomocysteinaemia plays a role in the early onset of atherosclerosis. Homocysteine is produced by the demethylation of the essential amino acid methionine. It is catabolized by cysthationine β-synthase, or is remethylated forming methionine, a reaction which in most tissues depends on the cofactor activity of folate and vitamin B_{12}.[43] Inherited defects result in severe hyperhomocysteinaemia, early onset of arteriosclerosis and frequent life-threatening thromboembolism. Several studies showed that moderate hyperhomocysteinaemia is common (25-42%) in patients with cerebrovascular and cardiovascular disease.[44-49] The increase in homocysteine in most instances is probably due to cysthationine β-synthase deficiency.[50] However, in a recent study no association of

homocysteine concentration with ischaemic heart disease could be detected.[51] Besides the possible atherogenic propensity, elevated homocysteine causes endothelial dysfunction. It enhances the activity of factor VII and factor V , and induces the expression of tissue factor.[52] Homocysteine also inhibits activation of protein C, both directly and by inactivating its vascular cofactor thrombomodulin.[53] Uncomplicated hyperhomocysteinaemia is not accompanied by a rise in thrombin potential (Bos et al., personal communication). Vitamin supplementation decreases plasma homocysteine concentrations. However, the clinical efficacy on atherothrombogenesis is not (yet) known.

Secondary prevention of stroke

Antiplatelet therapy is clinically important, but has rather limited efficacy in the prevention of vascular events (stroke, myocardial infarction, or death from a vascular cause). Antiplatelet therapy in patients with a prior stroke or transient ischaemic attack (TIA) results in an absolute risk reduction in non-fatal stroke of 2% (8.2% in treated patients, 10.2% in controls).[10 54] There has been some discussion on what dose of aspirin to use for the so-called secondary prevention of vascular events following TIA or stroke.[55 56] As no dose-effect response has ever been demonstrated clinically, the dose in use depends on various factors. In the Netherlands most patients receive low-dose aspirin treatment (30 mg a day), because a Dutch study showed no significant difference in efficacy of 30 mg or 283 mg of aspirin.[57]

In a recent study in which aspirin and oral anticoagulation were compared in non-cardioembolic stroke, the odds of dying from a bleeding complication was 20 times higher in the anticoagulated group than in the group on aspirin. Most of the bleedings were intracerebral haemorrhages.[11] Standard dose oral anticoagulation has only been proven beneficial in patients with cardioembolic stroke. The use of oral anticoagulants at very low intensity represents an attractive alternative in patients requiring long-term thrombosis prophylaxis. At least it seems likely that such a regimen would be associated with a lower bleeding risk than warfarin at standard intensity, and that it might also require less frequent laboratory monitoring. Treatment with fixed low-dose warfarin (1 mg/day) reduced the incidence of deep vein thrombosis after major gynaecological surgery,[12 13] but the combination of aspirin and fixed low-dose warfarin had no additional effect over aspirin alone in patients after myocardial infarction.[15] Low-intensity warfarin (INR below 2.0) had no effect in the prevention of stroke in patients with non-rheumatic atrial fibrillation.[14] Recently, it was shown that most patients can be stably anticoagulated with very low doses of warfarin, and that such regimens generally result in the suppression of baseline F_{1+2} levels, which is a marker

of prothrombin activation, by approximately 50%[58] and of factor VII coagulant activity.[59]

Erythrocyte aggregation

In states of no flow red cells in normal blood spontaneously adhere to form rouleaux. The large plasma proteins, of which fibrinogen is the most important, are responsible for generating the adhesive forces.[60] Other macromolecules, such as α2-macroglobulin and some immunoglobulins also contribute to red blood cell aggregation (RBCA).[61-63] Erythrocyte aggregates that are morphologically different from rouleaux may also occur. Aggregates have an important influence on blood rheology;[64] they increase blood viscosity at low shear rates and are largely responsible for the so-called viscoelastic properties of blood. RBCA is thought to endow blood with a yield stress which may decrease microcirculatory flow. RBCA occurs in states of low shear stress and, unlike platelet aggregation, is usually reversible. The extent of aggregate formation depends on the nature and the concentration of aggregating proteins, plasma viscosity, erythrocyte deformability, and hematocrit. RBCA is also influenced by erythrocyte surface charge density; negatively charged phospholipids may be exposed with a transbilayer movement of phosphatidylserine under certain circumstances.[65,66] In this way erythrocytes, like platelets, may develop procoagulant activity.

Enhanced RBCA increases blood viscosity in states of low shear rate diminishing blood passage through the cerebral capillary circulation.[6,67-72] Enhanced RBCA rather than hyperviscosity was responsible for the observed hypoxemic and neurologic symptomatology in an animal model.[64]

Hematocrit and plasma fibrinogen concentration are negatively associated with cerebral blood flow in stroke patients.[73] Enhanced RBCA may be one of the mechanisms to explain this finding. Several studies found enhanced RBCA in stroke patients.[6,71,74-78] There was a correlation between fibrinogen concentration, platelet activation and RBCA.[6,77] Erythrocytes can enhance platelet activity[79] and RBCA may secondarily exacerbate platelet aggregation.[80] Thrombospondin, one of the contents of the platelet α-granules, may also induce erythrocyte aggregation.[81] Consequently, enhanced RBCA could be caused by platelet activation.

The coagulation mechanism

Thrombin is the central enzyme in haemostasis and thrombosis.[82] Any type of thrombosis is essentially caused by thrombin, in an intricate collaboration with platelets. Thrombin acts at the level of the plasma, the platelet and the vessel wall. It is the most potent platelet activator and activated platelets are crucial in causing explosive thrombin generation. Thrombin itself is not

present in plasma, but can be formed by proteolytical cleavage of a precursor protein, prothrombin (factor II).

Tissue injury brings damage to the endothelium of the vessel wall, thereby leading to exposure of tissue factor (TF) to the plasma proteins. This TF binds to factor VII(a). Probably there is always some circulating factor VIIa to start the reaction. The resulting TF-factor VII complex activates factor X into factor Xa. Factor Xa catalyzes conversion of the TF-factor VII complex into the TF-factor VIIa complex, with a much higher enzymatic activity.[83] In addition, the TF-factor VIIa complex activates factor IX,[84-86] which in turn can also activate factor X into Xa.

Factor Xa alone can convert prothrombin into thrombin, although very slowly (less than 0.1‰ of the velocity of full prothrombinase). Small amounts of thrombin thus generated produce feedback reactions: activation of factor V into Va,[87 88] of factor VIII into VIIIa,[89 90] and activation of platelets. Activated platelets expose procoagulant phospholipids[91] to which factor Va can bind. After binding, factor Va serves as a platelet binding site for factor Xa. For rapid, massive generation of thrombin the full complex Va-Xa-phospholipids (prothrombinase) is required.

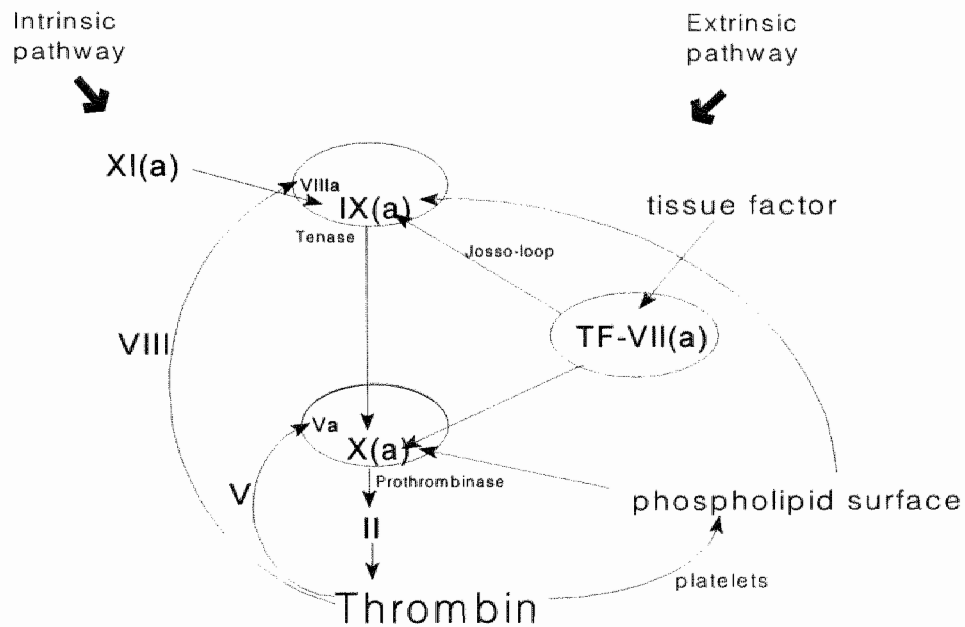


Figure 1.1. Sequence of activation of plasma coagulation proteins

Factor VIII circulates in the plasma attached to the very high molecular weight protein von Willebrand factor (vWF). Thrombin splits off a part of factor VIII, which causes the molecule to loose its affinity for vWF, after which it binds to phospholipids. Thereafter, factor VIIIa-phospholipid complex binds to factor IXa. The complex IXa-VIIIa-phospholipids (tenase) is capable of activating factor X, thus generating more prothrombinase. The formation of prothrombinase and tenase leads to a burst of thrombin generation by converting prothrombin into thrombin.

Another way to initiate blood coagulation, is via the so called contact activation reactions ('intrinsic pathway'). When blood comes into contact with negatively charged surfaces (like glass), factor XII, factor XI, prekallikrein and high molecular weight kininogen are activated, leading to activation of factor XI into XIa, which in turn activates factor IX. From this point the intrinsic and the extrinsic pathway merge. The role of the intrinsic pathway under physiological conditions has not been unequivocally clarified. On the other hand, feedback activation of factor XI by thrombin seems to be required for full thrombin development in platelet rich plasma (Keularts et al, personal communication).

To balance between haemorrhage and thrombosis thrombin formation has to be limited in space (see below) and in time. Limitation in time occurs via two mechanisms: activated protein C and tissue factor pathway inhibitor. Thrombin can bind to thrombomodulin, a membrane protein, present at the surface of intact endothelium. Thrombin adsorbs onto this protein and, in doing so, looses its procoagulant properties and activates protein C. Activated protein C, together with its cofactor protein S can inactivate the factors Va and VIIIa,[92] [93] in this way inactivating both tenase and prothrombinase. This will reduce the formation of thrombin.

Another regulation mechanism operates via tissue factor pathway inhibitor (TFPI), which binds to factor Xa.[94] The complex inhibits TF-factor VIIa complex. This mechanism ensures that tissue factor-induced factor X activation will stop as soon as sufficient factor Xa is present.

Thrombin, once formed, is inactivated by plasma protease inhibitors (antithrombins).[95] Antithrombin is the most important, contributing for 64% of total antithrombin activity, α2-macroglobulin accounts for 23%, and the remaining 13% is taken care of by other antiproteases (mainly α1-antitrypsin).[96] [97] The combined effect of these inhibitors makes the half life of thrombin in plasma 17 sec and that of (free) factor Xa 80 sec. Factor Xa in the prothrombinase complex is not affected by these antiproteases.

All of these mechanisms participate in the inactivation of thrombin, thereby contributing to the balance between thrombin formation and thrombin inactivation.

Platelets

The circulating platelet is a discoid cell, capable of very rapid responses to a range of physiological agonists (e.g. thrombin, collagen, ADP). On the platelet surface membrane glycoproteins (GP) are present, that function as receptors for adhesive proteins and physiological agonists. Collagen and thrombin are the primary, exogenous platelet activators. Fibrin also is a potent inducer of platelet procoagulant activity (fig.1.2).[98]

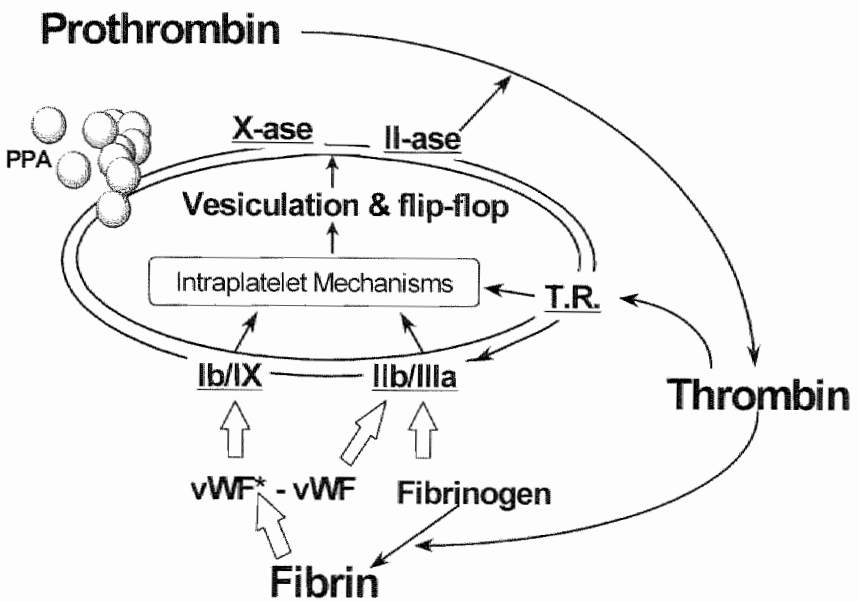


Figure 1.2. The role of platelets in thrombin formation. Thrombin, through interaction with a thrombin receptor (T.R.) induces the formation of functional glycoprotein IIb/IIIa receptors (activation of the platelet) that bind fibrinogen. Thrombin also forms fibrin from fibrinogen. Von Willebrand factor (vWF) adsorbs to fibrin and the interaction of platelet receptors glycoprotein Ib/IX with this vWF also activates the platelet. In the activated platelet procoagulant phospholipids become exposed. Also procoagulant microvesicles are shed from the platelet membrane: platelet derived procoagulant activity (PPA). Prothrombin activation takes place on these procoagulant surfaces (Figure by courtesy of S. Béguin, dep. of Biochemistry, University of Maastricht, The Netherlands).

The first phase of the haemostatic reaction is the adhesion of platelets to collagen in the exposed subendothelium of the vessel wall lesion. Platelets, bound to collagen, are readily activated by thrombin. Upon activation the platelet changes its shape from a smooth disc to a irregular sphere with extended pseudopods; in this way it can interact with other platelets. The

platelet releases the contents of the α-granules, which, among other things, contain factor V, fibrinogen, vWF, platelet factor 4, and thrombospondin. One of the provoked platelet reactions is the so-called 'flip-flop' of the membrane. The procoagulant phospholipids phosphatidyl serine (PS) and phosphatidyl ethanolamine (PE) are almost entirely located at the inside of the platelet membrane. Collagen and thrombin induce a transbilayer movement of phospholipids in this membrane,[66] resulting in an increased exposure of PS at the outer surface.[91 99 100] Following activation platelets thus provide the procoagulant surface necessary for activation of factor X and prothrombin.[101]

Another procoagulant reaction of the platelet membrane is the shedding of microvesicles. These phospholipid rich microvesicles expose PS at the outside and therefore can serve as a support for tenase and prothrombinase.[66 100 102 103] Also fibrin, even without thrombin being adsorbed to it, is a potent inducer of platelet procoagulant activity.[98 104]

Platelets adhere to collagen (in a wound) and fibrin (in a wound or thrombus). Platelets attached to this matrix become procoagulant. Platelets do not adhere to intact vessel wall; in this way thrombin generation is limited in space.

A recently formed haemostatic plug is unstable until it is consolidated by a fibrin network, which requires thrombin generation. So thrombin serves both as fibrin-forming enzyme, and as platelet activator. There are many interactions between the platelet and the coagulation proteins, e.g.:

1. Thrombin causes a procoagulant reaction in the platelet membrane. The platelets thus activated, foster thrombin generation.[82]
2. Fibrinogen serves as adhesive platelet protein as well as thrombin substrate.
3. vWF acts as a carrier protein for factor VIII, is crucial for platelet adhesion to subendothelium, and plays an important role in the generation of thrombin in platelet rich plasma.[105]

Fibrinogen

Fibrinogen is present in plasma in a concentration of 2-3 g/l, and in platelets (5-10% of the plasma content). In infections and other inflammatory states, the fibrinogen concentration in blood is elevated, most likely by increased synthesis.[106]

One of the effects of thrombin generation is the clotting of fibrinogen. Thrombin splits off two fibrinopeptides (A and B) from fibrinogen, and thus converts it into the fibrin monomer, which is the active form of fibrinogen.[106] Fibrin monomers spontaneously polymerize to form long fibrin strands. Factor XIIIa, activated by thrombin, also serves to crosslink the fibrin monomers to fibronectin and to crosslink fibronectin to collagen.

About 30-40% of the thrombin generated in plasma is incorporated into the generated fibrin, limiting the activity of thrombin.[98] [107] Fibrinogen is required for platelet aggregation.[108] The GPIIb/IIIa complex on the platelet surface membrane has been identified as the platelet fibrinogen receptor. This complex only forms in activated platelets. Upon activation, the receptor undergoes divalent cation dependent conformational changes and expresses fibrinogen binding sites.[109] The bound fibrinogen supports platelet aggregation by bridging adjacent platelets.

GPIIb/IIIa can also serve as a receptor for fibronectin, vWF and vitronectin.[110-113] However, fibrinogen remains the chief ligand, possibly because it is the most abundant.

From several epidemiological studies fibrinogen concentration has emerged as an important and independent risk factor for stroke and myocardial infarction.[114-119] Among stroke survivors hyperfibrinogenaemia was found to be an independent risk factor for subsequent cardiovascular events.[71] [120] Fibrinogen level also predicts the progression of atherosclerotic carotid stenosis.[121] In addition, fibrinogen concentrations are elevated in the presence of other known cerebrovascular risk factors such as smoking, hypertension, hyperlipoproteinaemia or diabetes.[122] Despite these associations fibrinogen concentration is an independent risk factor for cerebrovascular disease.

The mechanisms by which fibrinogen may promote atherosclerosis and thrombosis are diverse. Apart from its role in haemostasis, fibrinogen is the major determinant of plasma viscosity and induces reversible red blood cell aggregation. Both phenomena limit the fluidity of blood. High levels of fibrinogen and a high hematocrit are negatively associated with cerebral blood flow,[73] but not in all studies.[123] The haemorheologic consequences of hyperfibrinogenaemia might act at various levels: by reducing flow, by predisposing to thrombosis, and by enhancing atherogenesis.[124] Also, fibrin makes platelets procoagulant, and thus leads to formation of more fibrin.[104] This mechanism could possibly increase with higher fibrinogen, thus giving a novel explanation for the link between fibrinogen and stroke. Currently, a study is being done to investigate the effect of Ancrod®, a defibrinating enzyme, on reduction of fibrinogen, and outcome in stroke patients.

von Willebrand factor

The vWF is a polymeric plasma glycoprotein, which is synthesized in megakaryocytes and endothelial cells. It is found in plasma, in the subendothelium and in the platelet α-granules.[125]

vWF has three important functions in haemostasis:

1. It is the carrier for factor VIII, an essential cofactor in the generation of factor Xa.[126] [127] vWF protects factor VIII from inactivation by activated

protein C or factor Xa. If there is no circulating vWF, due to a congenital deficiency, there is no factor VIII. Infusion of vWF without factor VIII, makes factor VIII appear. So vWF is an absolute requirement for the maintenance of a normal level of factor VIII.

2. The vWF mediates platelet adhesion and aggregation.[128] It interacts with components of the subendothelium and with platelet receptors. The vWF promotes platelet interaction with the damaged vessel wall.[129-131] The involvement of vWF in mediating platelet adhesion and thrombus formation (i.e. platelet aggregation) is crucial under high shear stresses which are encountered in small vessels and stenosed arteries. Under conditions of high shear rates thrombus formation appeared to be more dependent on vWF than on fibrinogen.[132] vWF binds to endothelial components (e.g. collagen) that are exposed as a result of vessel injury. This adsorption induces a conformational change in the vWF molecule by which it acquires the property to interact with the platelets. GPIb-IX complex, one of the glycoproteins present on the platelet membrane, mediates the adhesion of unstimulated platelets to vWF which is bound within the subendothelium of damaged vessels.[133 134] The vWF can also bind to GPIIb/IIIa receptors.[134] This binding plays a role in platelet aggregation.[135] The vWF also mediates binding of platelets to fibrin through GPIb.[104 136-138]
3. A role of vWF recently discovered is that as a necessary mediator in the mechanism that brings out procoagulant activity in platelets. Thrombin induces platelet procoagulant activity in a GPIIb/IIIa dependent process. Fibrin requires GPIb for making the platelet procoagulant. VWF is a necessary cofactor in both mechanisms.[7 104] Inhibition of vWF or severe vWF deficiency prevents thrombin generation in platelet rich plasma almost completely.[105]

Several studies found a correlation between high levels of vWF and cerebrovascular disease.[8 9 139 140] Catto also found a relationship between vWF, stroke mortality and stroke type.[9] Previously, we found an elevation in vWF both in young stroke patients and in elderly stroke patients compared to controls. Elderly patients, however, had a much higher vWF concentration than young patients did (unpublished observation). This could be due to more extended endothelial damage related to large vessel atherosclerotic disease, which is more common in elderly than in young patients. As mentioned earlier however, vWF is not merely a marker of endothelial damage, but it also plays an active role in atherothrombogenesis.[141] Since vWF is important in developing platelet procoagulant activity, one can well imagine its possible role in adverse clinical events.

Plasma concentration of vWF is related to a number of other stroke risk factors, including smoking, hypertension, hyperhomocysteinaemia and diabetes.[142-145] There is controversy about the relation between vWF and age.[143 146] Like fibrinogen, vWF also is an acute phase reactant.

Thrombin generation

The enzymatic action of thrombin on a large number of substrates, and its role as inducer of the procoagulant effect in platelets is essential in haemostasis and thrombosis. The rise and fall of thrombin concentration after triggering coagulation in plasma results from the combined activity of the prothrombin activating enzyme prothrombinase and the thrombin inactivating processes, i.e. the binding of thrombin by antithrombins, such as antithrombin and α2-macroglobulin. The thrombin generation curve is one of the oldest tools of the coagulation trade. It is the basis of the so-called two-stage prothrombin estimation.[147-149] A typical thrombin generation curve is characterised by a lag-phase after which a burst of thrombin is seen that disappears due to the action of the natural antithrombins (fig. 1.3). Hemker and Béguin introduced the so-called *endogenous thrombin potential* (ETP), the area under the thrombin generation curve. This variable is the product of thrombin concentration, and the time that it acts.[97 150] It indicates how much substrate the thrombin generated in plasma can potentially convert. They also developed a method that allows a quantitative spectrophotometric measurement of thrombin generation, and hence of the ETP, both in platelet rich plasma and in platelet poor plasma.[151] The ETP gives information on the coagulation system as a whole, i.e. on the numerous pathways in which many positive and negative feedback loops interact. Consequently, measuring the ETP both in platelet rich plasma and in platelet poor plasma makes it possible to distinguish between the role of the platelets in interaction with the plasmatic coagulation proteins and the role of the plasmatic coagulation system.

Thrombin potential in platelet rich plasma

Thrombin formation and platelet activation are intimately linked in haemostasis and thrombosis. Thrombin can not be formed *in vivo* unless activated platelets expose their procoagulant phospholipid surface, whereas platelets have to be activated first by thrombin itself. Thrombin generation can be assessed in platelet rich plasma or whole blood. In this way, the interplay between blood platelets and the plasmatic coagulation factors under *in vitro* conditions that approach the *in vivo* conditions can be investigated.[152] Under these experimental circumstances procoagulant phospholipids are the rate limiting factor for thrombin generation and not the factors V or VIII.[151] Therefore, the moment of the burst of thrombin generation is the moment at which the platelets massively expose their procoagulant surface. The lag-phase can be shortened by adding a low amount of thrombin.

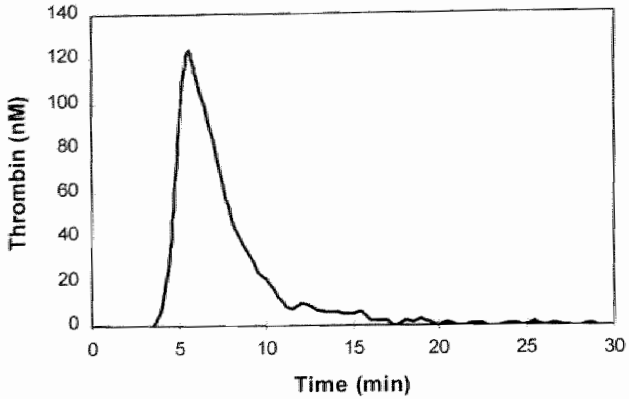


Figure 1.3. The thrombin generation curve in platelet rich plasma.

The course of events *in vitro* is as follows: freshly prepared platelet rich plasma is to be regarded as platelet poor plasma in which unactivated, hence non-procoagulant platelets are suspended. Coagulation is triggered by calcium in combination with a low concentration of tissue factor. Tissue factor induces the formation of small amounts of thrombin to be formed, which act on the platelets, that in this way will become activated. Once platelet activation reaches a (low) threshold value, thrombin generation accelerates, the platelets are activated en masse and provide the phospholipids that allows explosive thrombin generation, up to about 120 nM.

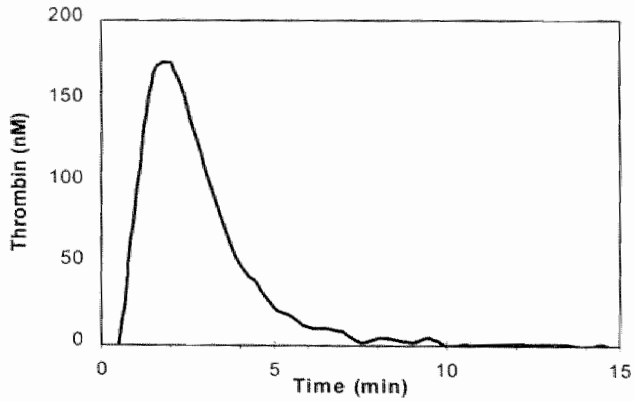


Figure 1.4. The thrombin generation curve in platelet poor plasma.

All antithrombotic medication that has proven to be active, i.e. heparin, oral anticoagulation and aspirin, cause an inhibition of the cooperative effect between platelets and thrombin, which is reflected in a prolongation of the lag-phase and a decrease of the ETP.

Thrombin potential in platelet poor plasma

In platelet poor plasma calcium, tissue factor and phospholipids are administered to trigger coagulation. Since there are no platelets present, phospholipids have to be added. This means that there is a very short lag-phase in platelet poor plasma (fig.1.4), whereas in platelet rich plasma the platelets need time to express their procoagulant phospholipid surface, resulting in a long lag-phase (circa 4 min.). In platelet poor plasma the ETP can also be continuously monitored.[150 153]

The protein C pathway is dependent upon the action of the thrombin-thrombomodulin complex. Thrombomodulin normally is linked to the vessel wall, and is not present in the plasma. Therefore, the activated protein C system is not active in isolated plasma. To reveal the role of the protein C pathway it is necessary to add thrombomodulin to the ETP assay. Resistance of factor V to activated protein C (factor V Leiden) and deficiencies of proteins C and S are risk factors for venous thrombosis, but they are not very likely to play a role in ischaemic stroke.[5 24 26-30]

The present study

Effective prevention of stroke is guided by the cause of stroke. In many young stroke patients the stroke cause remains uncertain. Conflicting data exist about hypercoagulability as a possible contributor to ischaemic stroke in young patients. Extensive laboratory screening for prethrombotic states in stroke patients is limited to the recognition of a number of known diseases that cause hypercoagulability, such as deficiencies of proteins C and S, factor V Leiden, hyperhomocysteinaemia etc. Such tests are expensive and not widely available. Besides, they often provide only negative information, because they may only exclude one specific known cause of hypercoagulability. Each of these tests is focused on only one protein of the coagulation system, and does not reflect the overall coagulation. Actual overall tests based on clotting time are not enough sensitive to hypercoagulability. Therefore, such tests have a low sensitivity, which makes them useless as a screening test for hypercoagulability that may antecede stroke in stroke survivors. Because the laboratory definition of prothrombotic diathesis is evolving rapidly and additional clinical studies continue to identify high-risk subgroups, it would be a great advantage to have a screening parameter of the coagulation system as a whole, i.e. the plasmatic

coagulation system together with the platelets. In this way further specific testing could be limited to patients with identified hypercoagulability. It was the main aim of this thesis to investigate whether the ETP can be used as such a screening parameter in young stroke patients. With the ETP, measured both in platelet rich plasma and in platelet poor plasma, it is possible to differentiate between hypercoagulability due to platelets, or hypercoagulability due to the plasmatic coagulation system. Also the influence of medication on the hypercoagulability can be studied with the ETP. Apart from cardioembolic stroke, secondary stroke prevention up till now is limited to antiplatelet therapy, which is only moderately effective. In specific subgroups other medication, like fixed low-dose oral anticoagulants, may be more effective in preventing vascular events. With the use of the ETP it may be possible to stratify young stroke patients in groups that will benefit from either oral anticoagulant or antiplatelet therapy.

Therefore, in this thesis we set out to explore the role of erythrocyte aggregation (chapter 2), the ETP in platelet poor plasma (chapter 3) and in platelet rich plasma (chapter 4) all in young patients with non-cardioembolic stroke. We also wanted to assess the influence of aspirin and fixed low-dose oral anticoagulants on ETP in healthy subjects and in young stroke patients (chapter 5).

Chapter 2

Enhanced red blood cell aggregation unrelated to fibrinogen: a possible stroke mechanism in young patients

Based on: Faber CG, Troost J, Vermes I, Lodder J, Kalsbeek-Batenburg EM, Kessels F, Haanen C. Enhanced red blood cell aggregation unrelated to fibrinogen: a possible stroke mechanism in young stroke patients. *Cerebrovasc Dis* 1997;7:70-76

Abstract

In many young stroke patients the cause of stroke remains unclear. Enhanced red blood cell aggregation is considered a factor related to the pathogenesis of stroke in elderly patients, in whom enhanced red blood cell aggregation is correlated with increased fibrinogen. We determined red blood cell aggregation, fibrinogen concentration, hematocrit value and erythrocyte sedimentation rate in 18 stroke patients ≤ 50 years of age in the early phase and in 40 stroke patients ≤ 50 years in the late phase, and compared the values to those in young control persons. We also determined these variables in stroke patients ≥ 60 years of age in the early and in the late phases and in elderly controls. In young stroke patients we found an enhanced red blood cell aggregation compared to young controls ($p < 0.00005$), both in the early and in the late phases, whereas fibrinogen was normal. Red blood cell aggregation was significantly associated with stroke after adjusting for fibrinogen, hematocrit and erythrocyte sedimentation rate (adjusted odds ratio 16.20; 95% confidence interval (CI) 2.80 - 93.61). Red blood cell aggregation was higher in elderly patients than in elderly controls ($p < 0.05$). In elderly patients fibrinogen was associated with stroke (crude odds ratio 12.92; 95% CI 2.54 - 65.82), whereas after adjusting for red blood cell aggregation, fibrinogen, hematocrit and erythrocyte sedimentation rate only erythrocyte sedimentation rate showed a significant association with stroke (odds ratio 26.37; 95% CI 1.93 - 359.74).

In conclusion: Enhanced red blood cell aggregation independently relates to stroke in young people, which may suggest that enhanced red blood cell aggregation contributes to stroke cause, whereas in elderly patients any such effect is probably related to confounding by raised fibrinogen.

Introduction

Despite extensive investigations the cause of stroke remains uncertain in many young patients.[2 18-20 154] In 1989, Tanahashi et al.[6] found enhanced red blood cell aggregation (RBCA) in elderly patients and considered this to be related to the pathogenesis of stroke. They showed a correlation between enhanced RBCA and increased fibrinogen, which in itself is known as a risk factor for stroke.[60 114 115] RBCA can also be influenced by the concentration of immunoglobulins.[62 63] Enhanced RBCA increases blood viscosity in states of low shear rate diminishing blood passage through the cerebral capillary circulation.[6 67-72 74] Therefore, enhanced RBCA may play a role in the cause of ischaemic stroke, specially among the young. We hypothesized that, if RBCA is an independent risk factor for stroke, an increased aggregation would occur more frequently among young patients with stroke compared to elderly patients and controls. We therefore studied RBCA in these three groups of subjects. We also determined fibrinogen concentrations, hematocrit values and erythrocyte sedimentation rates (ESR) as possible confounding factors.

Patients and Methods

The study population consisted of six groups. Groups I and II represented young stroke patients. Eighteen consecutive patients (11 men and 7 women) under age 50 with transient ischaemic attack (TIA) or stroke were included in group I. We investigated the blood of these patients in the early stroke phase (within 2 days after the ischaemic event). Median age was 39 years (range 23-48). Eight patients (44.4%) had hypertension, 3 patients (16.3%) suffered previous stroke. Group II consisted of 40 consecutive stroke patients, 23 men and 17 women, in whom blood was collected in the late phase (at least 2 months after the event, median 29 months, range 2-72 months). Fourteen of these patients were investigated also in the early phase. Median age at the time of stroke was 43 years (range 13-50). Of these patients, 2 (5%) had ischaemic heart disease, 20 (50%) had hypertension and 3 (7.5%) diabetes mellitus. Four patients (10%) suffered previous ischaemic stroke. Groups III and IV represented elderly stroke patients, aged over 60 years. Group III included 20 consecutive patients, 10 men and 10 women, investigated in the early stroke phase. Median age was 73 years (range 62-88). Ten patients (50%) had hypertension, 3 patients (15%) ischaemic heart disease, 4 patients (20%) diabetes mellitus and 4 patients (20%) suffered previous stroke. Group IV included 17 stroke patients, 6 men and 11 women, in whom blood was collected in the late phase (median 38 months after the event, range 2 - 108 months). Median age was 79 years (range 62-89). Of these

patients 4 (23.5%) were known with ischaemic heart disease, 6 (35.3%) had hypertension and 4 (23.5%) diabetes mellitus.

Group V included 34 young control persons, median age 31 years (range 21-49) and group VI 16 elderly control persons, median age 66 years (range 60-82).

Group I and III were prospectively enrolled between February 1992 and May 1993 at the Medical Spectrum Twente Hospital, Enschede. Group II was registered at the University Hospital Maastricht [155] and 14 patients of group I were also investigated in the late phase. Group IV was retrospectively registered at the Medical Spectrum Twente Hospital. Control subjects were all recruted at the latter hospital.

Patients with a potential source of cardioembolism and patients with intracerebral haemorrhage were excluded from the study.[23]

Investigations in all patients included standard blood tests, chest radiography, electrocardiography (ECG), non-invasive carotid studies and computer tomography of the brain. We carried out echocardiography, 24-hour ECG monitoring and cerebral angiography in selected patients.

We collected venous blood with minimal occlusion using ethylenediaminetetraacetic acid as anticoagulant and then determined RBCA photometrically in a transparant plate-cone chamber under controlled shear rate (MA1 Myrenne Aggregometer, Myrenne GmbH, Roetgen, Germany),[62 156] according to Schmid-Schönbein.[157] RBCA is expressed as extent of shear induced rouleaux formation in percent of normal (SIRF%). We determined whole blood and plasma viscosity in all young stroke patients under controlled shear rate (whole blood 11.5 s^{-1} and serum 230 s^{-1}) by a cone-plate viscometer (Digital Brookfield Viscometer type LVT-DVL-CT, Stoughton, Mass., USA) in the early phase (group I) and in the late phase (group II). Because the values for blood and plasma viscosity in young stroke patients appeared not statistically significantly different from those obtained in young control subjects, further measurements were not carried out in the older stroke patients. Plasma fibrinogen was determined according to the Clauss-Vermijlen-thrombin clotting time method (Schnitzer and Gross coagulometer, H. Amelung, Lemgo, Brake, Germany) using bovine thrombin (Topostasin). Hematocrit and haematologic indices were determined by an automatic cell counter (H1 Technicon Instrument Corp., Terrytown, N.Y., USA). We performed all tests in duplicate.

Apart from these tests we determined some other tests in all young stroke patients, like lupus anticoagulant, protein C and protein S measurements. In most patients (44 of 58 patients) also anticardiolipin antibodies were determined. We performed methionine loading test in 42 of 58 patients.

Statistical evaluation

Results are presented as median and range. We used Pearson's correlation coefficient to determine correlation, and the Mann-Whitney test to analyze differences in continuous variables between groups. We categorized continuous variables using cutoff points at the 33th and 66th percentile. Cutoff points were shifted untill each cell had 5 or more participants. If this procedure did not lead to cells with 5 or more participants, variables were dichotomized. We analyzed categorized variables in univariate analysis by means of crude odds ratios (OR) with 95% confidence intervals (CI). Multivariate logistic regression analysis was subsequently used to determine adjusted odds ratios with 95% CI with RBCA, fibrinogen, ESR and hematocrit as independent variables, and stroke as dependent variable.

Results

Median values and range of RBCA, fibrinogen, hematocrit and ESR in the different groups are shown in table 2.1.

Young patients

RBCA was significantly higher in young stroke patients both in the early and in the late phase, compared to young controls ($p< 0.00005$), whereas fibrinogen was not (fig. 2.1). ESR was higher compared to controls ($p=0.0004$). Hematocrit was normal. Young stroke patients had normal concentrations of immunoglobulins and normal values for medium and high shear viscosity. There was no difference between RBCA in the early or in the late phase. In the 34 patients with a territorial infarct (30 cortical, 4 brainstem) mean RBCA was 9.2, in 21 patients with a lacunar infarct mean RBCA was 10.9 and in 3 patients with TIA mean RBCA was 8.3 (table 2.2). RBCA was higher in lacunar than in cortical strokes, but not statistically significant (relative risk 2.53, 95% CI 0.80 - 7.95).

After adjusting for fibrinogen, hematocrit and ESR, RBCA was significantly associated with stroke in young persons (adjusted OR 16.20; 95% CI 2.80 - 93.61, fig. 2.2). There was an association between stroke and the intermediate category of hematocrit values (hematocrit 0.42 - 0.46, adjusted OR 8.70; 95% CI 1.74 - 43.46). There was no significant association between stroke and ESR or fibrinogen concentration. There was no influence of sex. Measurements in haemostasis, like protein C and S, revealed no abnormalities. Anticardiolipin antibodies were all negative. RBCA was not significantly higher in 7 patients with abnormal methionine loading test than in 35 patients with normal methionine loading test.

Table 2.1. Median values (range) of red blood cell aggregation, fibrinogen, hematocrit and erythrocyte sedimentation rate in stroke patients and control subjects.

	Young patients		Young controls	Elderly patients		Elderly controls
	early phase (n=18)	late phase (n=40)	(n=34)	early phase (n=20)	late phase (n=17)	(n=16)
RBCA, % SIRF	9.4 (5.8-13.5)	9.8 (5.8-18.3)	7.2 (3.3-9.1)	9.7 (4.4-14.9)	9.6 (7.3-11.8)	8.6 (5.4-10.8)
Fibrinogen, g/l	3.5 (2.0-6.2)	3.1 (2.0-6.0)	3.3 (2.5-4.5)	4.3 (2.6-7.2)	4.2 (2.9-6.2)	3.4 (2.9-4.1)
Hematocrit	.46 (.36-.52)	.43 (.30-.50)	.45 (.37-.54)	.43 (.36-.50)	.41 (.36-.51)	.48 (.40-.50)
ESR, mm in 1 h	7 (2-64)	8 (2-79)	4 (2-12)	22 (5-66)	23 (4-106)	9 (5-16)

Table 2.2. Frequency of various stroke types in young patients

	Young stroke patients, n	RBCA (mean)
territorial infarct	34	92
lacunar infarct	21	109
TIA	3	83
Total	58	

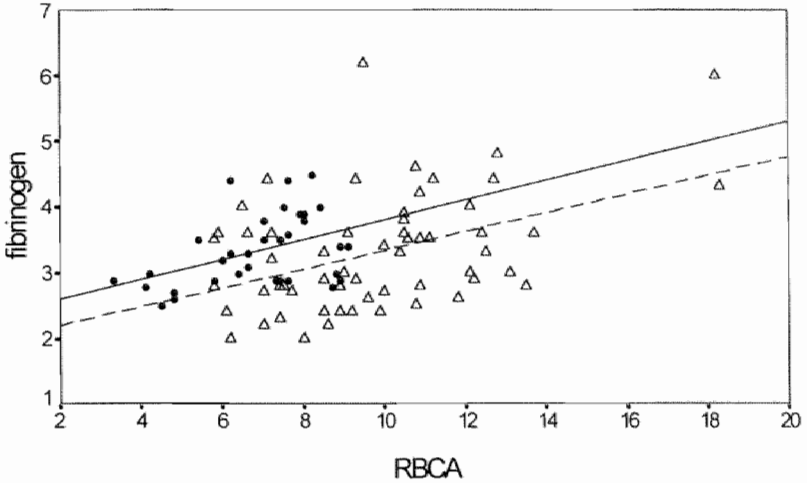


Figure 2.1. RBCA and fibrinogen in young stroke patients (△) compared with young controls (•). The regression lines of fibrinogen on RBCA are drawn for patients (-----) and for controls (——).

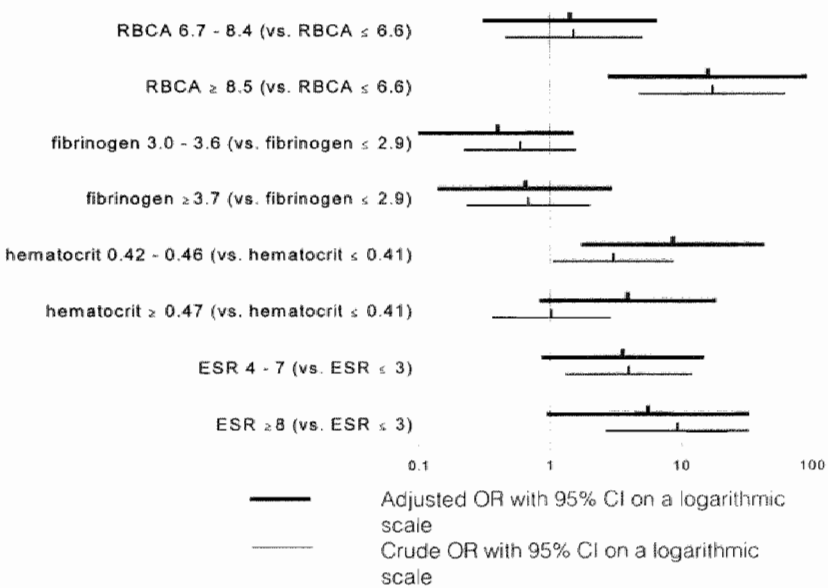


Figure 2.2. Comparison between young stroke patients and controls by bivariate and multiple logistic regression. An OR>1 indicates that the variable is associated with stroke. When the 95% CI crosses the vertical '1' line, the OR is not significant.

Elderly patients

RBCA was higher in elderly stroke patients compared to elderly controls ($p<0.05$), as were fibrinogen ($p<0.00005$, fig. 2.3) and ESR ($p<0.00005$). Hematocrit values did not differ between groups. After adjusting for RBCA, fibrinogen, hematocrit and ESR only the ESR showed a significant association with ischaemic stroke in these patients (adjusted OR 26.37; 95% CI 1.93 - 359.74, fig. 2.4). Without adjusting for these variables there was an association between fibrinogen and stroke (crude OR 12.92; 95% CI 2.54 - 65.82), and RBCA and stroke (crude OR 6.33; 95% CI 1.11 - 36.0). After adjusting for fibrinogen, the association between RBCA and stroke is no longer significant. There was no association between hematocrit and stroke. A scatterplot of the values for RBCA in the different groups is presented in figure 2.5.

Discussion

Among young stroke patients we found an enhanced RBCA not determined by abnormal concentrations of fibrinogen, immunoglobulins or abnormal hematocrit values. Our groups were too small to allow a logistic model with various additional variables that could have influenced the results, like stroke subtype, vascular risk factors, or haemostatic parameters. However, various

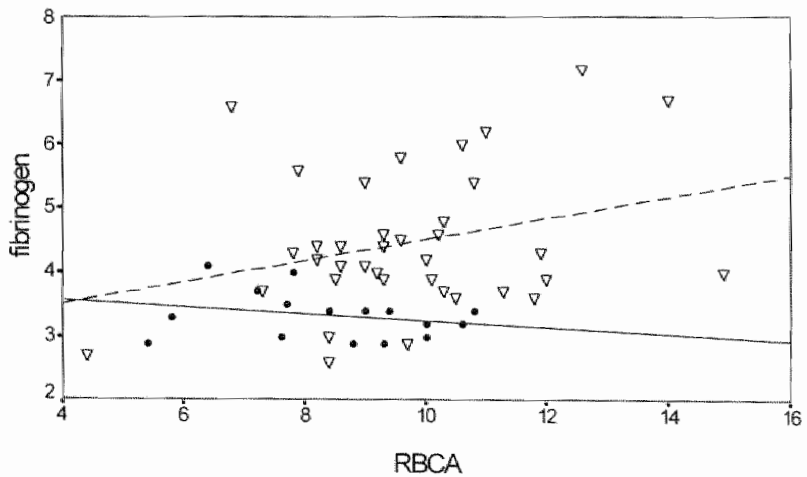


Figure 2.3. RBCA and fibrinogen in elderly stroke patients (▽) compared with controls (•). The regression lines of fibrinogen on RBCA are drawn for elderly patients (----) and for controls (——).

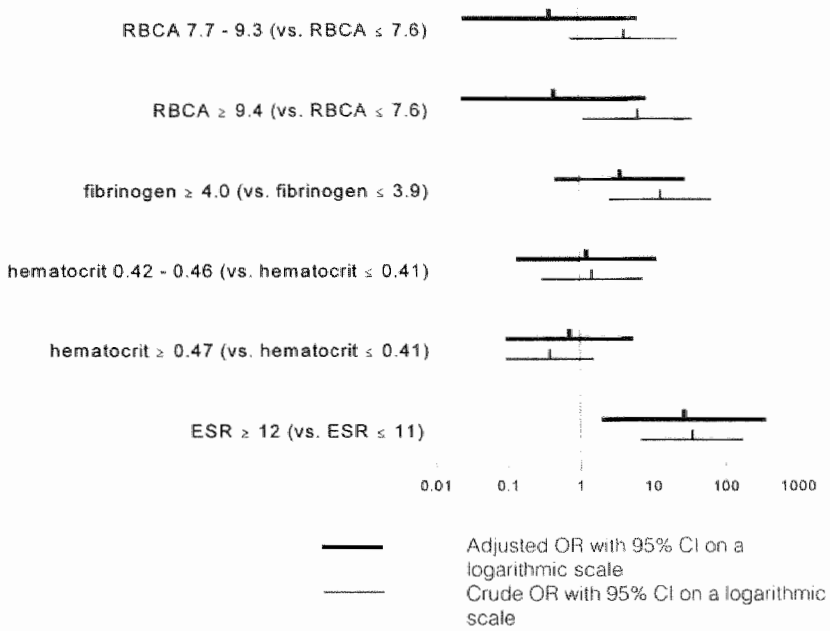


Figure 2.4. Comparison between elderly stroke patients and controls by bivariate and multiple logistic regression. An OR >1 indicates that the variable is associated with stroke. When the 95% CI crosses the vertical '1' line, the OR is not significant.

factors influencing haemostasis (protein C, protein S, lupus anticoagulant) were normal. Especially during the early phase following stroke, RBCA might have been influenced by various factors. However, it is less likely that stroke would permanently alter RBCA. Our findings late following stroke may therefore indicate that enhanced RBCA may contribute to the cause of stroke in some young patients. This concurs with the suggestion of Ernst et al.,[71] that enhanced RBCA as a chronic rheological disorder may increase the risk of a recurrent ischaemic event.

RBCA measured *in vitro* may not reflect the situation *in vivo*. However, haemorheological factors may influence cerebral blood flow.[73 80 158] Furthermore, in arteries and veins filled with irregular aggregates of erythrocytes in macroglobulinemic mice, abnormal flow through pial vessels was observed, whereas enhanced RBCA was considered responsible for the observed neurological symptoms in these animals.[67] However, although our findings suggest that enhanced RBCA may act as a cause of stroke, our study design does not permit definitely such a conclusion.

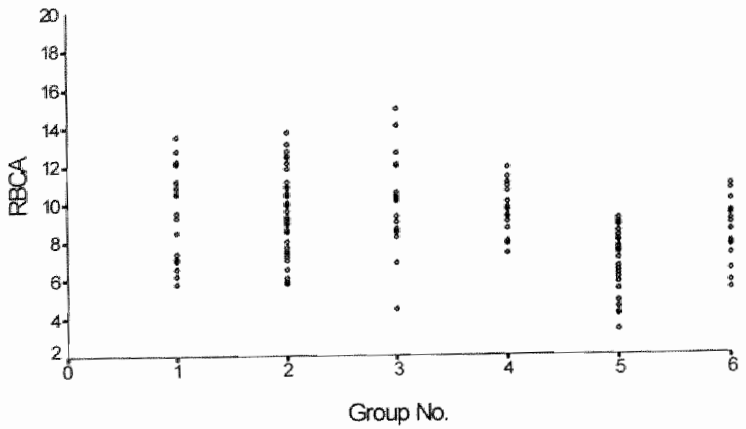


Figure 2.5. Scatterplot of the values for RBCA in the different groups. Group 1 = young stroke patients in the early stroke phase; group 2 = young patients in the late stroke phase; group 3 = elderly stroke patients in the acute stroke phase; group 4 = elderly stroke patients in the chronic phase; group 5 = young controls; group 6 = elderly controls.

The physiological importance of RBCA is its tendency to increase the blood viscosity in low shear flow and to disturb the passage in cappillary circulation through the formation of sludge.[159] Especially after a poststenotic pressure drop shear stresses are diminished and RBCA might be enhanced. Small arteriolar stenoses are often found in lacunar disease. We found enhanced aggregation more frequently among young patients with lacunar than with cortical stroke, but, probably due to small numbers, this difference was not statistically significant. Others also mentioned enhanced RBCA in patients with lacunar stroke.[74] Whether enhanced RBCA occurs more frequently in patients with small vessel disease than in those with large vessel disease remains to be investigated.

Most of our young stroke patients had a normal hematocrit and therefore were classified in the intermediate category of hematocrit value, which explains the association between stroke and the intermediate category of hematocrit value in these patients.

Elderly patients had a slightly higher RBCA than controls, but multivariate regression analysis showed no independent correlation between enhanced RBCA and stroke in this group, due to a strong association with fibrinogen concentration. Fibrinogen is known as a risk factor for vascular disease,[114,115] and is associated with an enhanced RBCA.[6]

Fibrinogen and ESR are strongly correlated; ESR will be high if fibrinogen is raised. Crude OR for fibrinogen in our series was high, but after

multivariate logistic regression analysis with RBCA, ESR, fibrinogen and hematocrit as independent variables only the adjusted OR for ESR remained significant.

We investigated young stroke patients registered prospectively in the past,[155] who were able and willing to come to the hospital, and therefore included functionally less affected patients. In case of a relationship between the enhanced RBCA and degree of neurological deficit, any relationship would have been towards an underestimation of the strength of association between the rheological disorder and stroke. We consider a selection bias towards the opposite direction less likely.

For our statistical analysis of continuous variables we chose cutoff points at the 33th and 66th percentile. Changing cutoff points did not essentially change the outcome. Furthermore, shifting cutoff points until each cell had 5 or more participants was done to prevent empty cells in the multivariate regression analysis and thereby extremely high odds ratios.

A cause for the enhanced RBCA in young stroke patients was not found. May be some plasma protein, other than fibrinogen, could be responsible for the enhanced RBCA. This would be of future interest.

In conclusion: In young patients RBCA may independently contribute to stroke cause, whereas in elderly patients any such effect is probably related to confounding by raised fibrinogen.

Chapter 

Thrombin generation in platelet poor plasma of young stroke patients

Abstract

Haemostatic abnormalities may contribute to the development of stroke in young patients. We used the thrombin potential, an overall indicator of the plasma coagulability, to determine whether young stroke patients have abnormalities in the plasmatic coagulation system. To investigate whether there were any disturbances in the protein C pathway, we also added thrombomodulin to the thrombin generation assay. We determined the extrinsic and the intrinsic thrombin potential in 41 young stroke patients (≤ 50 years) and in 70 healthy control persons. Based on the 33th and 66th percentile of the extrinsic thrombin potential values, patients were divided into three categories. We found a plasma based hypercoagulability in about one third of young stroke patients. Patients with recurrent stroke had a significantly higher thrombin potential than other patients (p=0.03). After the addition of thrombomodulin five patients had an insufficient inhibition of thrombin generation, indicating an abnormality of the protein C pathway.

In conclusion: With the use of the thrombin potential in platelet poor plasma as a screening parameter of the plasmatic coagulation system, we identified hypercoagulability in approximately one third of young stroke patients. Patients with recurrent stroke have a higher thrombin potential than those without. Abnormalities in the protein C pathway are easily detectable with the thrombomodulin test, and may contribute to the development of stroke in the presence of other vascular risk factors. In patients with an identified hyperactive coagulation system, further investigations into the cause of this plasma based thrombotic tendency are warranted.

Introduction

Thrombin represents the end product of numerous enzymatic reactions in the clotting system. The production of thrombin is controlled by inhibitory reactions due to plasma antiproteases (e.g. antithrombin and α_2-macroglobulin) and negative feedback reactions, initiated by thrombin itself, as in the protein C pathway. When too much free thrombin is generated, a thrombotic tendency occurs (hypercoagulability). There are many laboratory tests that may point at an underlying specific cause of such hypercoagulability, among which are the determination of the various clotting factors. A single laboratory parameter that is increased in all forms of hypercoagulability would be very useful for the detection of plasma based thrombotic tendency. The time integral of thrombin formation, i.e. the endogenous thrombin potential (ETP), is an overall indicator of the plasma coagulability, and might therefore be a good candidate for detecting abnormalities in the coagulation system. The thrombin potential is known to be elevated in patients with antithrombin deficiency (about 40%), deep venous thrombosis (about 29%), in patients with ischaemic heart disease (about 10%)[160] and in women using oral contraceptives (about 17%).[161 162]

Until now, in many young stroke patients the underlying cause of stroke remains unclear. In up to a quarter of young stroke patients a haematologic abnormality may contribute to the development of stroke.[5 24 25] Antithrombin deficiency, protein S deficiency, protein C deficiency, antiphospholipid antibodies, and activated protein C resistance are said to be related to stroke at young age, but their quantitative contribution to the development of stroke is at least doubtful.[5 24 26-30] In some of these disorders the ETP is increased.[160]

Disorders in the protein C pathway increase thrombin formation by interfering with the mechanism that down-regulates the action of prothrombinase. This pathway is initiated by the interaction of thrombin with thrombomodulin (TM). The thrombin-TM complex activates protein C. Activated protein C (APC), together with protein S, inactivates factors Va and VIIIa and consequently shuts down further thrombin generation. This mechanism is impaired in congenital deficiency of proteins C and S or, more frequently, by resistance of factor V to the action of protein C. The latter disorder can be either congenital,[163-165] or acquired, e.g. by the intake of oral contraceptives.[162] Disorders of the protein C pathway cause a slight, but significant elevation of the ETP (of about 10%).[161 162] In the presence of added TM (or APC) in healthy controls a decrease of the ETP is observed. In the case of APC resistance of factor V, the decrease of the ETP is significantly less pronounced after addition of TM or APC.[166]

Thrombomodulin is normally linked to the vessel wall. Therefore, it is impossible to define the 'physiological concentrations' in a soluble reaction

mixture as we use for the ETP. The ETP without TM and with an optimal concentration of solubilized TM obviously represent the two extremes of the physiological situations. We therefore tested the ETP in the extrinsic system with and without added soluble TM.[167] To reveal disorders in the mechanism that is dependent upon the factors VIII, IX or XI we also determined the ETP in the intrinsic system. Under physiological circumstances these factors come into play at low tissue factor concentrations, but *in vitro* their activity is best revealed in a contact-activated system (i.e. intrinsic pathway).

Patients, materials and methods

Patients

There were 41 consecutive patients under age 50 with ischaemic stroke (three patients had a TIA), between July 1986 and July 1996, and 70 healthy volunteers. Age and sex were similar in both groups. After approval of the medical ethical committee, written informed consent was obtained, both from patients and controls. Routine investigations in the patient group included standard blood tests, electrocardiogram, brain CT, non-invasive carotid studies and echocardiography. Cerebral angiography was performed in 21 cases for specific individual reasons. Clinical characteristics of patients and controls are listed in table 3.1 and 3.2. All patients were investigated at least 3 months after the ischaemic event, to exclude the influence of acute phase reactions on the measurements.

Patients with a specific stroke cause, such as a potential source of cardioembolism or an arterial dissection, and patients with intracranial haemorrhage were excluded from the study.

Materials

Plasma

Blood was collected on 0.13 M trisodium citrate; nine parts of blood to one part of citrate solution. An open (non-vacuum) venipuncture was performed in order to avoid platelet activation. The first ml of blood was discarded to get rid of venipuncture related tissue factor. Samples were centrifuged twice at 1000 x g, at 15°C for 10 min. This plasma was stored at -80°C.

Normal pool plasma was obtained in the same way from at least 10 healthy donors, and pooled. The pool was centrifuged twice at 1000 x g, at 15°C for 10 min and stored in 1 ml aliquots at -80°C.

Table 3.1. Clinical characteristics of young stroke patients and controls.

	patients n=41	controls n=70
age (mean ± SD)	43.9 (± 6.0)	39.5 (± 8.9)
range	25-50	23-57
sex	19 male (46.3%)	33 male (47.1%)
Medical history		
Prior stroke/TIA	4 (9.8%)	0
Myocardial infarction	3 (7.3%)	0
Angina pectoris	5 (12.2%)	0
Peripheral artery disease	4 (9.8%)	0
Hypertension	19 (46.3%)	3 (4.3%)
Hypertensive medication	12 (29.3%)	0
Chronic obstructive pulmonary disease	3 (7.3%)	3 (4.3%)
Diabetes mellitus	2 (4.9%)	0
Hypercholesterolaemia	12 (29.3%)	1 (1.4%)
Vascular family history	24 (58.5%)	33 (47.1%)
Oral contraceptive use	9 (22.0%)	10 (14.3%)
Smoking	28 (68.3%)	20 (28.6%)
Use of alcohol	16 (39.0%)	58 (82.9%)
Alcohol units/week (among users)	12.4 (± 10.7)	8.1 (± 9.3)
Stroke type		
Cortical	23 (56.1%)	
Lacunar	9 (22.0%)	
Cerebellar	1 (2.4%)	
Brainstem	5 (12.2%)	
TIA	3 (7.3%)	

Reagents

- Ancrod, a fibrinogen clotting enzyme of the Malayan Pit Viper, was obtained as the commercial preparation Arwin® (Knoll AG, Ludwigshafen, Germany).
- Buffer A consisted of 20 mM Hepes-NaOH, 150 mM NaCl, 0.5 g/l Bovine Serum Albumin (BSA; Lot: A-7030, Sigma), pH 7.35.
- Relipidated recombinant tissue factor preparations, in which phospholipids are present, were a gift of Dade Productions (Düdingen, Switzerland).

Table 3.2. Data on routine laboratory investigations in 41 young stroke patients

		normal values
Hemoglobin (mmol/l)	8.93 (± 0.98)	8.2-11.0
Hematocrit (l/l)	0.42 (± 0.05)	0.40-0.52
Leucocytes (x 10^9/l)	7.91 (± 2.18)	3.5-11.0
Thrombocytes (x 10^9/l)	290.3 (± 88.7)	130-350
Cholesterol (mmol/l)	6.0 (± 1.2)	4.1-6.4
Triglycerides (nmol/l)	1.93 (± 0.92)	0.80-1.94
Number of patients with:		prevalence in the normal population
Anticardiolipin antibodies	2 (4.9%)	7-8%[39]
Lupus anticoagulant	1 (2.4%)	1-2%[53]
Hyperhomocysteinaemia	6 (14.6%)	5-7%[52]

- Phospholipids (Avanti Polar Lipids, Alabama, USA) consisted of phosphatidyl serine (PS), phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) in proportions of 20, 60 and 20%, respectively. These phospholipids were used in a solution of 5.28 µM. In the thrombomodulin test the final concentration was 1.1 µM.
- SQ68 (Diagnostica Stago, Boehringer, Mannheim) was used as substrate for thrombin in the intrinsic pathway.
- For the extrinsic pathway and the thrombomodulin test we used MSC Val Arg pNA (MSCV), a novel chromogenic substrate synthesized in our laboratory and described in detail elsewhere.[160 168] For the details of the determination of the ETP with different substrates, see Hemker (1993) or Wielders (1997).[150 160] Here it suffices to say that the optical density signal with MSCV is 1.97 than that obtained with SQ68.
- As a trigger for the extrinsic pathway we used recombinant tissue factor in a concentration of about 30 pM. For the intrinsic pathway we used Actin FS® (Dade Productions, Düdingen, Switzerland), a suspension of ellagic acid and soy bean phosphatides. Actin FS® was diluted 1:2.5.
- Thrombomodulin (Kordia, Leiden, The Netherlands) was used in a final concentration of 30 nM.

Methods

Defibrination of plasma

To allow measurements of optical density, plasma had to be defibrinated. Defibrination was achieved by coagulation of 1 ml plasma with 1 U Ancrod. The samples were incubated for 10 min at 37°C, allowing the formed fibrin to

polymerize, then kept on ice for 10 min. The resulting clot was removed from the plasma by winding it out on a small plastic spatula. It has been determined that this procedure does not activate the clotting system.[150]

Measurement of thrombin generation in PPP

Thrombin generation in PPP was measured using continuous monitoring of thrombin formation, as described earlier.[150] This method, in which a slow reacting thrombin substrate was used, has been adapted for high throughput screening on a Cobas centrifugal analyzer.[160] This automaton can measure 28 samples simultaneously. The volumes used were 80 µl of defibrinated plasma and 20 µl of recombinant tissue factor solution (for the extrinsic pathway) or 20 µl of Actin FS® solution (for the intrinsic pathway). After 30 sec of incubation, which is sufficient for temperature equilibration, thrombin generation was started by adding 20 µl of a prewarmed start solution containing 0,1 M $CaCl_2$ and 3 mM of substrate. After the start of the reaction the optical density at 405 nm was recorded at intervals of maximally 30 sec for at least 15 min. For data handling the centrifugal analyzer was connected to a personal computer.

Calculation of the thrombin potential

The thrombin potential can not be obtained directly from the optical density that develops during the experiment, because thrombin is not the only enzyme to convert the substrate. Part of the free thrombin is irreversibly bound to α_2-macroglobulin to form a complex that retains amidolytic activity. The course of optical density in time that is experimentally obtained has to be split in the part due to the activity of free thrombin and the part due to the α_2-macroglobulin-thrombin complex by a mathematical procedure.[160] The thrombin potential is expressed as a percentage of the ETP in normal pool plasma.

Thrombomodulin test

This test is based on the extrinsic thrombin generation test in which the amount of tissue factor is reduced from 15 to 0.15 ng. Thrombin bound to thrombomodulin activates protein C. Then this serine protease will inactivate the factors Va and VIIIa and consequently will reduce the thrombin formation, thus the ETP will decrease. In this way it is possible to investigate the function of the protein C pathway. To 75 µl defibrinated plasma was added 25 µl of a solution containing: 5.28 µM phospholipids (PS, PC and PE, see the materials section), recombinant tissue factor (about 7.2 pg) and 720 µM thrombomodulin. After 30 sec of incubation at 37°C in the Cobas machine, thrombin generation was started by adding 20 µl of a prewarmed solution containing 0,1 M $CaCl_2$ and 3 mM of chromogenic substrate (MSCV). The final concentration of phospholipids was 1.1 µM, of thrombomodulin 30 nM

and of recombinant tissue factor 0.15 ng. With this concentration of tissue factor the clotting time in glass was 60 sec. The thrombomodulin concentration was choosen to obtain an inhibition of around 40% of the ETP of the normal pool plasma. The result of the thrombomodulin test is expressed as a percentage of the inhibition obtained by the addition of thrombomodulin to normal pool plasma, and was determined in 37 patients and in 24 controls.

Statistical evaluation

Results are presented as median and the 25 and 75 percentiles. We used the Kendall's tau to determine correlation, and the Mann-Whitney test to analyze differences in continuous variables between groups. Patients were categorized in groups using cutoff points at the 33th and 66th percentile of the value of the ETP. In the thrombomodulin test we considered a percentage inhibition below the 5th percentile of the control group (this was a percentage inhibition of less than 57% of the ETP after addition of thrombomodulin) as an insufficient reaction to thrombomodulin. We analyzed the association between stroke and the thrombomodulin test by means of odds ratios (OR) with 95% confidence intervals (CI) with a logistic regression model.

Results

Median values (25-75 precentile) of the ETP in the extrinsic and intrinsic pathway and of the thrombomodulin test are shown in table 3.3. The Mann-Whitney test did not show a significant difference between ETP values in young stroke patients and controls.

Table 3.3. Median values (25-75 percentile) of ETP in the extrinsic and intrinsic pathway, and of the thrombomodulin test (TM) in young stroke patients and in controls.

	Young stroke patients n=41	Controls n=70
ETP extrinsic	106 (93-121)	105 (98-113)
ETP intrinsic	105 (87-122)	100 (91-110)
TM	81 (62- 97)*	79 (68- 88)*

* determined in 37 patients and in 24 controls (see the methods section)

The patients were categorized, using cutoff points at the 33th and 66th percentile of the values of the ETP (table 3.4). Numbers of patients and controls (in percentages) in these three categories are shown in fig. 3.1. There was a significant correlation between ETP in the extrinsic and in the intrinsic pathway (Kendall's tau 0.57, $p<0.001$) (fig.3.2). Patients with peripheral artery disease (n=4) had higher ETP compared with other patients (extrinsic 123 (111-136), intrinsic 133 (116-168), $p=0.03$ and $p=0.02$, respectively). Patients who suffered a recurrent stroke (n=8) also had a significantly higher ETP in the extrinsic pathway ($p=0.03$) (table 3.5). Using the Mann-Whitney test no significant differences were found between patients with or without ischaemic heart disease, hypertension, hyperhomocysteinaemia, diabetes or current smoking. A significant difference was found in ETP in the extrinsic and in the intrinsic pathway between males and females. Females had a lower ETP, both in the control and in the patient group ($p<0.01$).

There were 5 patients with an insufficient inhibition of the ETP after addition of thrombomodulin. These patients showed an inhibition of 13, 32, 45, 48 and 56%, respectively. The values of the thrombomodulin test for patients and controls are shown in figure 3.3. In a logistic regression model an insufficient inhibition in the thrombomodulin test was significantly associated with stroke (odds ratio 9.9; 95%CI 1.1-87.6). Patients with an insufficient inhibition in the thrombomodulin test were all females. Four of these patients had a normal ETP. Three of these patients used oral contraceptives at the time of the ischaemic event (all patients stopped oral contraceptive use after their stroke), and four smoked cigarettes. Two patients had a hyperhomocysteinaemia.

Table 3.4. Results in median (25-75 percentile) of ETP in the extrinsic and in the intrinsic pathway, and of the thrombomodulin test (TM) in three categories based on the extrinsic ETP values in patients and in controls.

	Group 1 (ETP ≤ 96)		Group 2 (ETP 97-117)		Group 3 (ETP ≥ 118)	
	Controls n=15	Patients n=14	Controls n=45	Patients n=13	Controls n=10	Patients n=14
ETP extrinsic	87 (78-92)	89 (86-93)	105 (101-113)	106 (101-114)	121 (119-122)	122 (121-127)
ETP intrinsic	78 (70-90)	83 (74-94)	102 (93-111)	105 (96-108)	111 (96-115)	128 (121-145)
TM	61 (53-83)	80 (67-87)	78 (71- 82)	75 (58- 96)	93 (83-100)	93 (65-111)

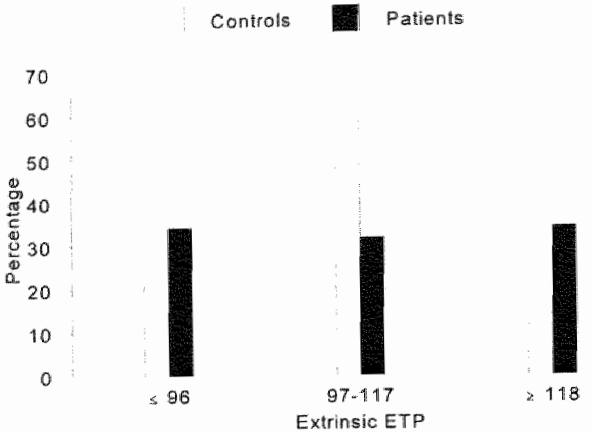


Figure 3.1. Percentages of extrinsic ETP values in the three categories (based on 33th and 66th percentile) in patients and controls. Patients are more present both in the lower and in the higher range, whereas the controls are mostly in the middle range of the ETP values .

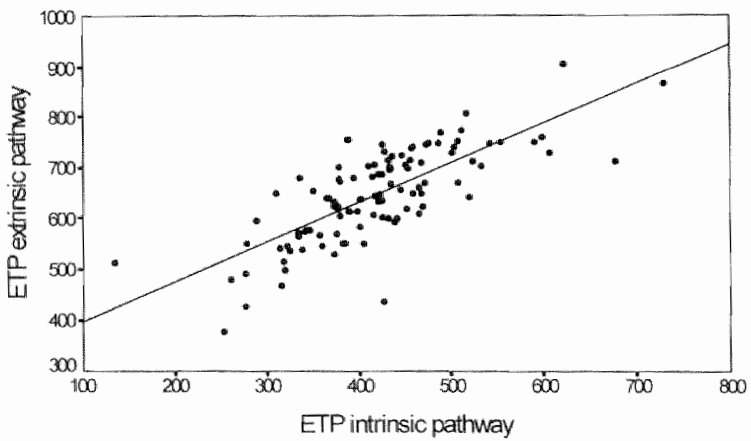


Figure 3.2. Correlation of the ETP in the extrinsic and in the intrinsic pathway in young stroke patients and in controls. The regression line of extrinsic on intrinsic ETP is drawn. There is a highly significant correlation between the extrinsic and the intrinsic ETP.

Table 3.5. Median values (25-75 percentile) of ETP in extrinsic and intrinsic pathway and of the thrombomodulin test (TM) young stroke patients without and with recurrent stroke (RS).

	Patients without RS n=33		Patients with RS n=8		p-value
ETP extrinsic	102	(90-121)	119	(110-136)	3
ETP intrinsic	102	(83-120)	116	(101-143)	NS (0.0502)
TM	81	(62- 97)	78	(60-100)	NS

Discussion

With the use of the ETP in PPP as a screening parameter of the plasmatic coagulation system, we identified hypercoagulability in approximately one third of young stroke patients. A striking finding was that patients with recurrent stroke had significantly higher ETP than those without. This could indicate that patients with a plasma based hypercoagulability have a higher risk of developing recurrent stroke. Stroke probably is multicausal in origin and it may be more appropriate to think of hypercoagulability as one of the possible contributing causes, or, in other words, as a risk factor. Coagulopathy as contributor to the development of stroke in the young may vary from 1-25%, but a definite relationship with stroke remains unclear.[5 24 25 169 170] The question to what extent an existing hypercoagulability is a risk factor for the development of stroke is difficult to answer as long as the definition and detection of hypercoagulability are limited to a relatively small number of known diseases that cause hypercoagulability, such as deficiencies of proteins C and S, factor V Leiden, anticardiolipin antibodies and hyper-homocysteinaemia. Most of these disorders are unlikely to play a quantitative role in the development of stroke.[5 24 26-30 39] As yet unidentified hypercoagulable states undoubtedly account for many other prethrombotic states.[171] The ETP offers the possibility to detect abnormalities in the haemostatic function of the blood, and appears to be rather sensitive. ETP does not allow identification of a specific underlying coagulation disorder, as it is an overall test of the coagulation system. Therefore, what caused the plasma based hypercoagulable state in our young stroke patients is not yet clear. Abnormalities in the protein C pathway can not be held responsible for the observed hypercoagulability in our patients, because their thrombomodulin inhibition was normal. ETP increases with the use of oral contraceptives,[161] whereas sensitivity to activated protein C decreases.[162] Because all patients stopped taking the contraceptive pill after their stroke, oral contraceptive use is not likely to be the cause of our findings.

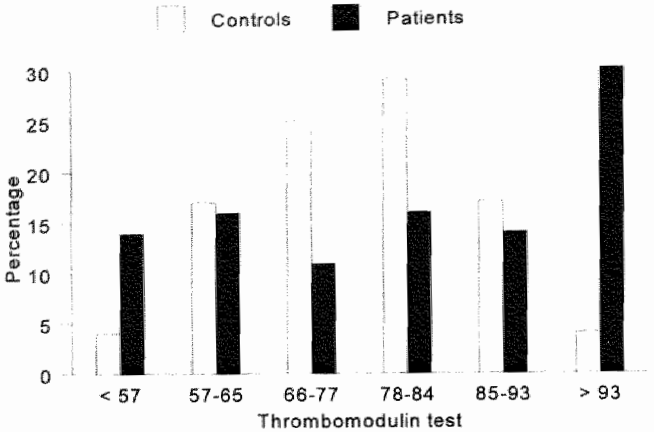


Figure 3.3. Percentages of controls and patients in subgroups, according to the value of the thrombomodulin test (in percentage inhibition). From this figure it is clear that the values of the thrombomodulin test in the controls show a normal distribution pattern, whereas the patients have more values both in the higher and in the lower range.

With the standard thrombin generation test to which thrombomodulin is added, the function of the protein C pathway can be explored. From previous experiments we know that percentages of inhibition in the thrombomodulin test in patients with protein C deficiency, protein S deficiency, or factor V Leiden are low (40±9%, 23±9%, 56±18%, respectively, versus 81±16% in controls) (Wielders, personal communication 1998). An insufficient decrease of the ETP after addition of thrombomodulin allows detection of an abnormality in the protein C pathway. This pathway plays an important role in the inactivation of thrombin. Consequently, an abnormality in the protein C pathway could induce a prethrombotic state.[30 172 173] Therefore, our five patients with an insufficient response to thrombomodulin probably had a prethrombotic state. Abnormalities in the protein C pathway are especially associated with venous thrombosis, but the precise role of protein C or protein S deficiency or of factor V Leiden as contributors to stroke is uncertain.[24 26 172 174] Deficiency of protein C or protein S, or resistance to activated protein C (factor V Leiden) have been linked with stroke in many case reports.[173 175-181] Currently it is believed that these disorders are not the primary cause of stroke, but rather enhance an already existing increased stroke risk.[24 27 29 30 53 182 183] Thus, a thrombotic event may result from the convergence of an (inherited) predisposition to thrombosis (like protein C deficiency or factor V Leiden) with an acquired thrombogenic stimulus (e.g. atherogenic risk factors).[31 171 184] All our five patients with an abnormal

thrombomodulin test had one or more additional vascular risk factors at the time of the stroke (smoking (n=4), oral contraceptive use (n=3), hyperhomocysteinaemia (n=2)). This concurs with the findings that factor V Leiden increased the risk of myocardial infarction in women, whereas this increased risk was largely confined to current smokers.[32]

Our study could be criticized for the reason that we performed our measurements following and not prior to stroke: a haematologic abnormality identified after stroke did not necessarily antedate the stroke, and can be a consequence rather than the cause of stroke. However, support for an antecedent, causal role of the haematologic disorder includes its persistence in subsequent months. For this reason we tested our patients at least 3 months after the ischaemic event. Therefore, we believe that our findings are not merely a consequence of stroke, but that many patients under 50 with cryptogenic stroke have hypercoagulability that may play an important role in the development of stroke. This idea is supported by the finding that high ETP is associated with recurrent stroke. Further study may also identify a role of ETP detected hypercoagulability in elderly patients, in whom atherosclerosis is the most likely stroke cause.

In conclusion: In a subgroup of young stroke patients hypercoagulability can be identified via the thrombin generation test. Patients with recurrent stroke had a higher ETP than those without. Abnormalities in the protein C pathway, as detected by the thrombomodulin, test are more common in young stroke patients than in controls. These protein C pathway abnormalities may contribute to the development of stroke, especially in the presence of other vascular risk factors. In patients with an identified hyperactive coagulation system, further investigations into the cause of this plasma based thrombotic tendency are warranted.

Chapter 

Thrombin generation in platelet rich plasma, platelet derived procoagulant activity, and von Willebrand factor in young stroke patients

Abstract

How often coagulation disturbances are the cause of stroke in young people remains unclear, as reported percentages vary from one to 25%. We used the thrombin potential (the time integral of thrombin formation) in platelet rich plasma to determine whether young stroke patients have a hypercoagulability. Comparing the thrombin potential in platelet rich plasma to the thrombin potential in platelet poor plasma allows determination of platelet related or plasma based hypercoagulability. Therefore, we determined the thrombin potential in platelet rich plasma in 41 young stroke patients and in 70 controls. The von Willebrand factor is a necessary mediator in the mechanism that brings out procoagulant activity in platelets. We therefore also determined platelet derived procoagulant activity and von Willebrand factor. The thrombin potential in platelet rich plasma was significantly higher in young stroke patients than in controls ($p=0.002$). Also platelet procoagulant activity and von Willebrand factor were significantly higher in patients than in controls ($p=0.045$ and $p=0.0006$, respectively). After excluding patients with a plasma based hypercoagulability, patients were divided in three groups on the basis of thrombin potential values in platelet rich plasma. High thrombin potential was significantly associated with stroke, both in the group with the intermediate and in the group with the highest thrombin potential (odds ratio 5.1; 95% CI 1.8-15.1, and 3.7; 95% CI 1.3-10.3, respectively). In a linear regression model von Willebrand factor was associated with platelet procoagulant activity and with the thrombin potential, whereas platelet procoagulant activity also was associated with the thrombin potential.

In conclusion: Thrombin generation in platelet rich plasma is elevated in young stroke patients. Comparing the thrombin potential in platelet rich plasma and in platelet poor plasma allowed us to identify patients with platelet related hypercoagulability. Platelet derived procoagulant activity and von Willebrand factor are increased in young stroke patients and both are related to the thrombin potential in platelet rich plasma. This indicates that among the numerous causes that may be behind an increased thrombin potential, a high concentration of von Willebrand factor is of significant importance. Our findings provide a pathophysiological explanation for the epidemiological observation that relates an increased concentration of von Willebrand factor to the occurrence of stroke.

Introduction

In chapter 3 we observed that in about one third of young stroke patients thrombin generation in platelet poor plasma is enhanced. Thrombin generation in platelet poor plasma is a laboratory test, created to assess the role of plasmatic clotting factors in establishing a hypercoagulable (or in other patients groups a hypocoagulable) state. *In vivo* thrombin is generated in blood, and the blood cells play an important role in thrombin generation. By far the most important blood cells in this respect are the platelets. Therefore, thrombin generation in platelet rich plasma (PRP) is one step nearer the physiological reality than thrombin generation in platelet poor plasma. Thrombin, already at low concentrations (about 5 nM), activates platelets and makes them expose procoagulant phospholipids that in turn facilitate the formation of thrombin by providing the procoagulant phospholipid surface necessary for activation of factor X and prothrombin.[66 91 100 101] Measurement of the endogenous thrombin potential (ETP; i.e. the area under the thrombin generation curve) in platelet rich plasma (PRP) and in platelet poor plasma allows determination of hypercoagulability, and differentiates between abnormalities in the plasmatic and the cellular coagulation system. Not only the coagulation factors and the blood platelets, but also von Willebrand factor (vWF) is pivotal in thrombogenesis.[7 104] vWF is a necessary mediator in the mechanism that brings out procoagulant activity in platelets. Thrombin induces platelet procoagulant activity in a GPIIb/IIIa dependent process. Fibrin requires GPIb for making the platelet procoagulant. VWF is a necessary cofactor in both mechanisms.[7 104] In several studies high vWF was associated with cerebrovascular disease.[8 9 139 140] We hypothesized that increased vWF could play a role in the development of stroke because, by enhancing platelet procoagulant activity, it would induce higher thrombin generation, i.e. hypercoagulability. If this hypothesis would be correct, a correlation between vWF and ETP in PRP would be expected, and (a number of) young stroke patients would show high values of both vWF and ETP. Therefore we determined the ETP in PRP and the concentration of vWF in young stroke patients, and in controls. As a correlate to thrombin generation we also measured platelet derived procoagulant activity in serum, i.e. the amount of phospholipid microvesicles, shed by activated platelets.

Patients, materials and methods

Patients

There were 41 consecutive patients under age 50 with an ischaemic stroke and 70 healthy volunteers. Precize clinical data of patients and controls are described in chapter 3.

Materials

Plasma
Blood was collected on 0.13 M trisodium citrate; nine parts of blood to one part of citrate solution. An open (non-vacuum) venipuncture was performed in order to avoid platelet activation. The first ml of blood was discarded to get rid of venipuncture related tissue factor. PRP was obtained by single centrifugation at 250 x g, at 15°C for 10 min. The platelet count was adjusted to 300.000/µl with homologous platelet poor plasma (centrifuged for 10 min at 1000 x g). Plastic tubes and pipettes were used throughout so as to minimize contact activation. All experiments in PRP were carried out within 60 min of venipuncture. Experiments in patients and controls were carried out in parallel.

Serum
After the thrombin generation test was performed the reaction mixture was put on ice, and subsequently centrifuged at 15.000 x g for two min. The supernatant was stored at -70°C.

Reagents for thrombin generation
- Buffer A consisted of 20 mM Hepes-NaOH, 150 mM NaCl, 0.5 g/l Bovine Serum Albumin (BSA; Lot: A-7030, Sigma), pH 7.35.
- Buffer B was the same as buffer A with 20 mM EDTA, pH 7.9.
- Chromogenic substrate used for thrombin was S2238: H-D-Phe-Pip-Arg-pNA.2HCl.
- The trigger used for coagulation was 0.1 M $CaCl_2$ with recombinant tissue factor in a concentration of 0.0075 ng/ml; with this concentration of recombinant tissue factor the clotting time of PPP in plastic tubes is 8 min.
- Stopping fluid was 1 M citric acid.

Methods

Measurement of thrombin generation in PRP
Thrombin generation curves were obtained as described in detail by Béguin and Hemker.[96 151] In short, 480 µl PRP is incubated at 37°C with 120 µl of Buffer A until temperature equilibration (5 min). Coagulation was initiated at

t=0 by adding 120 µl 0.1 M $CaCl_2$ with recombinant tissue factor. The reaction mixture was continuously stirred by a small spatula and 10 µl subsamples were drawn at equally spaced intervals of 30 sec and diluted in prewarmed (37°C) cuvettes containing 490 µl of Buffer B with 200 µM S2238. The reaction was stopped after 2 min by adding 300 µl of 1 M citric acid. As soon as the reaction mixture coagulated, the clot was wound on the spatula and removed. The optical density (OD) was read at 405 nm. The spectrophotometer as well as the subsampling and stopping pipette were connected to a personal computer that calculated the increase in OD per minute from the OD and the moment of subsampling and stopping. The amidolytic values were converted into nM thrombin via a standard calibration curve of active site titrated human α-thrombin. The lag time of thrombin formation is defined as the moment at which the thrombin concentration rises from a 0-5 nM level to a 10 nM level.

Calculation of the thrombin potential

The ETP is defined as the area under the thrombin generation curve.[82 96 150 160] The ETP is the product of thrombin concentration, and the time that it acts.[97 150] It indicates how much substrate the thrombin generated in plasma can potentially convert. The ETP can not be read directly from the optical density that develops during the experiment, because thrombin is not the only enzyme that converts the substrate. Part of the free thrombin is irreversibly bound to α2-macroglobulin, thereby losing its biological, but not its amidolytic activity. Therefore, the course of the optical density has to be divided in the part due to the activity of free thrombin, and the part due to the α2-macroglobulin-thrombin complex. Because the velocity of the formation of the latter complex is proportional to the amount of free thrombin, the ETP can thus be calculated by a mathematical procedure.[153] The ETP in PRP is expressed in percentages of the mean value in the control group.

Platelet derived procoagulant activity in serum

The serum which was obtained from the reaction mixture after the thrombin generation test was performed, was diluted 6.67 times in buffer A (18 µl serum: 102 µl buffer A). 50 µl of subsample was incubated for 4 min at 37°C with an assay mixture, containing 0.87 nM factor Xa, 21 nM factor Va and 24 mM $CaCl_2$ in buffer A. At t=0 50 µl prothrombin (6 mM) was added. After 4 min, 10 µl of subsample was taken to a cuvette containing 465 µl buffer B. The amount of thrombin formed was calculated from the continuous measurement of absorbance change at 405 nm after the addition of 25 µl of S2238 (4mM). The amount of thrombin formed depends on the activity of prothrombinase. The activity of prothrombinase is dependent on the amount of procoagulant phospholipids. These phospholipids are exposed by microvesicles, shed by the activated platelets in the reaction mixture.

Therefore the amount of thrombin formed is a measure of the platelet derived procoagulant activity.
Platelet derived procoagulant activity was determined in 34 patients and 52 controls.

von Willebrand factor
vWF antigen was determined with an enzyme linked immuno-absorbent assay (ELISA) kit from Diagnostica Stago, Boehringer, Mannheim. vWFwas determined in all patients and in 65 controls.

Statistical evaluation
Variables are presented as median and 25 and 75 percentile. As discussed in chapter 3, 14 of the 41 patients had an elevation of ETP in platelet poor plasma (PPP), so in these patients an abnormality of the plasmatic coagulation system was suspected (fig 4.1). The remaining patient group (n=27) was categorized into three groups using cutoff points at the 33th and 66th percentile of the ETP in PRP. We used the Mann-Whitney test to analyze differences in distribution of continuous variables between groups. We analyzed relationships between ETP in PRP, platelet derived procoagulant activity, vWF and fibrinogen using linear regression analysis. To check whether relationships were different for patients and controls, we also performed linear regression analysis separately for patients and controls. Results of linear regression analysis are presented in regression coefficient with 95% confidence intervals (95% CI). We analyzed the association between stroke and ETP in PRP by means of odds ratios (OR) with 95% confidence intervals (CI) with a logistic regression model.

Results

Median values (25, 75 percentile) of ETP, platelet derived procoagulant activity, vWF, fibrinogen and antithrombin III of the patients and of the controls are shown in table 4.1. The ETP in PRP is expressed in percentage of the control group (median 515 (25, 75 percentile: 449, 553)). The ETP in PRP was significantly higher in young stroke patients than in controls (p=0.002). Also platelet derived procoagulant activity (p=0.045), vWF (p=0.0006) and fibrinogen (p=0.0007) were higher in patients than in controls.

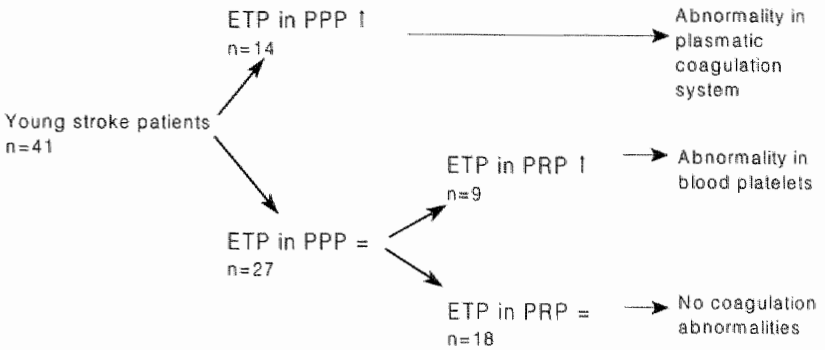


Figure 4.1. Flow diagram of thrombin potential (ETP) in platelet poor plasma (PPP) and in platelet rich plasma (PRP) (↑ : enhanced; = : normal).

Patients with no abnormalities in the ETP in platelet poor plasma (n=27) were divided in three groups on basis of the ETP values (33th and 66th percentile). The results of these separate groups are shown in table 4.2. The group with the highest ETP (group 1) had (of course) a significant higher ETP (p=0.0003), but also a higher vWF (p=0.0119), platelet derived procoagulant activity (p=0.0082), and fibrinogen (p=0.0223) than controls. In the group with a moderate increase in ETP (group 2) both ETP and vWF were higher than in controls (p=0.0009 and p=0.0403, respectively). Platelet derived procoagulant activity in this group was higher, though not statistically significant. The group with the lowest ETP in PRP (group 3) did not differ from the controls. With the combined results of the ETP in PPP (chapter 3) and in PRP patients can be divided into a group with suspected abnormalities in the plasmatic coagulation system (n=14), a group with platelet related hypercoagulability (n=9), and a group with no coagulation abnormalities (n=18) (fig.4.1).

ETP in PRP was significantly associated with stroke, both in the group with intermediate and high ETP (odds ratio 5.1 and 3.7, 95% CI 1.8-15.1 and 1.3-10.3, respectively). In a regression model with ETP as dependent and vWF as independent variable, vWF was significantly associated with the ETP (fig.4.2; table 4.3) In a regression model, platelet derived procoagulant activity was strongly associated with the ETP (fig.4.3). Fibrinogen level was not associated with the ETP in PRP. In a regression model with platelet derived procoagulant activity as dependent variable, vWF was a significant predictor of elevation of platelet derived procoagulant activity. There were no

Table 4.1. Median values (25-75 percentiles) of ETP in PRP, platelet procoagulant activity, vWF, fibrinogen and antithrombin III in young stroke patients and controls

	Young stroke patients n= 41	Controls n=70	p-value
ETP in PRP	111 (104-188)	102 (89-110)	0.002
platelet procoagulant activity	90 (69-106)*	79 (65- 88)*	0.045
vWF	102 (87-118)	86 (74-100)*	0.0006
fibrinogen	3.2 (2.8- 4.0)	2.8 (2.5- 3.2)	0.0007
antithrombin III	106 (98-109)	108 (104-111)	NS

* not determined in all subjects, see the methods section

significant differences between patients with territorial and lacunar infarcts, with respect to ETP in PRP, platelet derived procoagulant activity and vWF. There was no relationship between ETP in PRP and age, hypertension or other vascular risk factors.

Discussion

The ETP is an overall assay of the blood coagulation system. With the use of the ETP in PRP, the interplay between blood platelets and the plasmatic coagulation factors under *in vitro* conditions that approach the *in vivo* conditions, can be investigated.[152] Measuring the ETP in PRP and in PPP allowed us to exclude hypercoagulability in about 50% of young stroke patients, thereby limiting the need for further extensive laboratory investigations in search for specific coagulation abnormalities in these

Table 4.2. Results in median (25-75 percentiles) of ETP in PRP, platelet derived procoagulant activity (PPA), vWF and fibrinogen in the patient subgroups and in controls.

	Controls n=70	Group 1 (ETP ≥ 118) n=9	Group 2 (ETP 111-117) n=10	Group 3 (ETP ≤ 110) n=8
ETP in PRP	102 (89-110)	124 (119-134)	113 (111-115)	94 (81-103)
PPA	79 (65-88)*	107 (78-117)*	91 (72- 99)*	50 (45-100)*
vWF	86 (74-100)*	102 (92-138)	100 (91-113)	97 (73-124)
fibrinogen	2.8 (2.5- 3.2)	3.3 (3.0- 3.8)	3.5 (2.7- 3.9)	2.9 (2.2- 3.2)

* not determined in all subjects, see the methods section

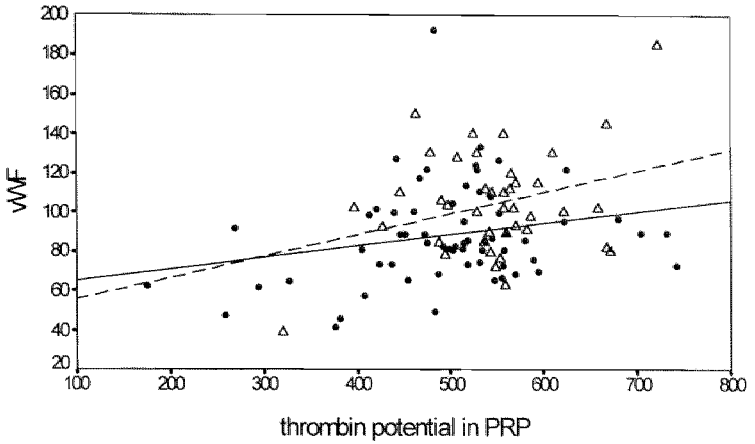
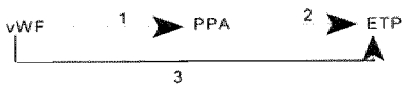


Figure 4.2. ETP in PRP and vWF in young stroke patients (△) and controls (●). The regression lines of vWF on the ETP are drawn for patients (----) and for controls (—).

Table 4.3. Results of linear regression analysis (regression coefficient (95% CI)) of ETP in PRP, platelet derived procoagulant activity (PPA) and vWF in controls, in young stroke patients, and in the whole group of subjects.

	Regression model		Controls	Patients	Overall
1	dependent	PPA	0.16	0.23	0.23
	independent	vWF	(-0.06-0.39)	(-0.07-0.53)	(0.05-0.40)*
2	dependent	ETP	2.07	2.05	2.11
	independent	PPA	(0.83-3.31)**	(1.08-3.02)***	(1.32-2.89)***
3	dependent	ETP	0.99	0.91	1.16
	independent	vWF	(-0.03-2.03)	(0.02-1.79)*	(0.49-1.84)***



Presumed relationships between vWF, PPA and ETP in PRP.

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

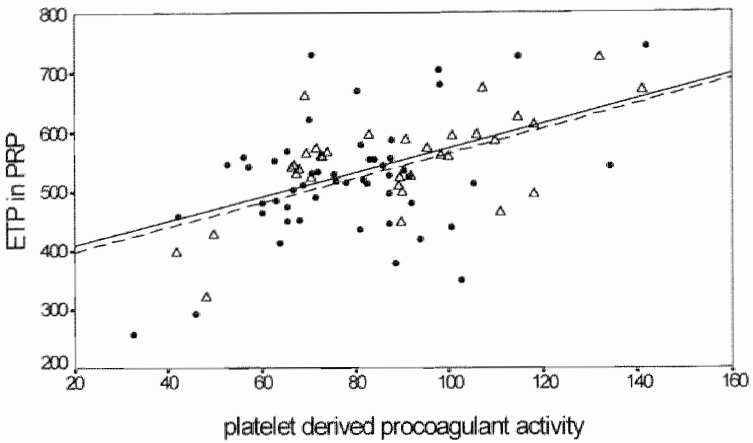


Figure 4.3. ETP in PRP and platelet derived procoagulant activity in young stroke patients (△) and in controls (●). The regression lines of platelet procoagulant activity on the ETP are drawn for patients (----) and for controls (—).

patients. With the ETP it is possible to establish any coagulation abnormality, and to differentiate whether such abnormality is due to the plasmatic coagulation system or related to the blood platelets. More than half of our patients appeared to have a hypercoagulability. In about one third of the patients the hypercoagulability was likely to be due to an abnormality in the plasmatic coagulation system (chapter 3), whereas in about a quarter it was likely to be induced by the blood platelets (fig 4.1). We found an enhanced ETP in PRP of young stroke patients when compared to controls. High ETP in PRP (both the intermediate and the high values) appeared to be a predictor for stroke. Our results indicate a far more important role of coagulation disorders in the development of cryptogenic stroke in young patients than so far assumed. With the ETP it is possible to identify coagulation abnormalities, and to differentiate whether such abnormality is due to the plasmatic coagulation system or related to the blood platelets. Once this is known, one is able to choose a more rational appraoch to secondary thrombosis prevention, i.e. antiplatelet or anticoagulant therapy. Furthermore, a high ETP in platelet poor plasma indicates that it is worthwile to search for abnormalities in the plasmatic coagulation system, whereas a high ETP in PRP suggests searching in the as yet hardly explored field of platelet based hypercoagulability.

When a haematologic abnormality is identified after stroke, the haematologic disorder can not be assumed to have antedated the stroke, and can be a consequence rather than the cause of stroke. Support for an antecedent, causal role of the haematologic disorder includes its persistence

after the acute phase of stroke. For this reason we tested our patients at least 3 months after the ischaemic event. Therefore, we believe that our findings reflect the premorbid state of the patients and allow the conclusion that hypercoagulability plays an important role in the development of stroke in a significant number of patients.

Measurement of thrombin formation in PRP is highly informative because it includes the influence of the blood platelets and therefore represents the situation *in vivo* better than thrombin generation in PPP does. However, other blood cells, the vessel wall, and flow characteristics which are present *in vivo*, do not play a role in the thrombin generation test in PRP. Nevertheless, the ETP reveals coagulation abnormalities in young stroke patients that are not detected by other tests. In its present form however, it is not an easy test that can be applied on a routine basis. One test requires two working hours by experienced technicians.

Under the experimental circumstances of the thrombin generation test in PRP procoagulant phospholipids are the rate limiting factor for thrombin generation and not the factors V or VIII.[151] Therefore, the moment of the burst of thrombin generation is the moment at which the platelets massively expose their procoagulant surface. In this process platelets also shed procoagulant microvesicles, that remain in the serum after the experiment. The coupling of these processes is reflected by the correlation between ETP in PRP and platelet derived procoagulant activity, as seen in our patients. It is not a priori clear in what way the cause-effect relationships between thrombin generation and platelet derived procoagulant activity go. In those patients with a high ETP in PPP, the high levels of thrombin may promote high levels of platelet derived procoagulant activity. This mechanism may also play a role in PRP, but there the reverse reaction may be more plausible: platelets that shed more procoagulant microvesicles may cause more thrombin to be generated. Therefore, increased platelet derived procoagulant activity may be the cause of hypercoagulability in a subgroup of young stroke patients. Other investigators have also reported a sustained increase in platelet activation in patients with cerebral ischaemia.[169]

Currently it is unclear what aberrations of the intraplatelet mechanisms lead to an increased procoagulant function of the platelet. Platelet activation is an extremely complicated process[185-188] in which many functions are possible sites of congenital or acquired disturbances. From the type of studies as reported here, it may in the future be possible to define individuals or families in whom specific pathogenic mechanisms can be identified.

A factor contributing to the procoagulant reaction of the platelet, is vWF. vWF mediates the interaction between fibrin and platelets through GPIb[136-138] and this interaction is one of the pathways that makes platelets procoagulant.[104] Binding of vWF to GPIIb/IIIa receptors on the platelet membrane plays a role in platelet aggregation[128,135] and also contributes to

the procoagulant reaction of the platelet.[7] So, vWF appears as a necessary mediator to express platelet procoagulant activity via GPIIb/IIIa as well as via GPIb and fibrin, and turns out to be indispensable for thrombin generation in PRP.[7] Earlier studies demonstrated a correlation between vWF and cerebrovascular disease.[8 9 139 140] Catto also found a relationship between vWF, stroke mortality and stroke type (higher level of vWF among patients with large vessel disease).[9] vWF was increased in our young stroke patients. We also found a significant correlation between ETP in PRP and vWF, and between platelet derived procoagulant activity and vWF. Obviously, vWF is not merely a marker of endothelial damage, but plays an essential role in coagulation through its effect on platelet activation. Our findings therefore provide a pathophysiological explanation for the epidemiological observation that relates increased concentration of vWF to the occurrence of stroke and to the increased mortality in stroke survivors.[8 9]

Fibrinogen is an independent risk factor for stroke and myocardial infarction.[114-116] Fibrin induces platelet procoagulant activity, which in turn leads to production of more thrombin and eventually to the formation of even more fibrin.[98 104] This circle may be more vicious when fibrinogen level is high, which might explain the link between fibrinogen and stroke. Fibrinogen was elevated in our young stroke patients, but there was no correlation between fibrinogen and ETP in PRP. Absence of fibrinogen raises the thrombin generation curve in PPP.[98] This can be explained by the absorption of thrombin by the fibrin formed during coagulation. During a thrombin generation experiment the clot is wound on a spatula and removed, thereby reducing the effect of fibrin on the thrombin generation. This may be one of the reasons that we did not find a relationship between fibrinogen and ETP in PRP.

In conclusion: Thrombin generation is elevated in young patients with cryptogenic stroke. In about a quarter of the patients the hypercoagulability is related to the blood platelets, and determination of the ETP in PRP can be used to detect such platelet related hypercoagulability. Platelet derived procoagulant activity and vWF are increased in young stroke patients and both are related to ETP in PRP. This identifies a high concentration of vWF as an important one among the numerous possible causes for increased thrombin generation in PRP.

Chapter 

Influence of aspirin and fixed low-dose oral anticoagulation on thrombin generation in young stroke patients and in healthy volunteers

Abstract

Antiplatelet therapy has limited efficacy in the secondary prevention of stroke. Therefore, there is an urgent need for a more potent therapeutic modality. It would be advantageous if patients with hypercoagulability either due to the plasmatic coagulation system or related to platelets could be separately identified, as this may eventually allow a more rational prescription of a preventive drug. It is conceivable that a decrease in the thrombin generation, induced by aspirin or fixed low-dose oral anticoagulants, may lower the risk of thrombotic events in patients. Therefore, we determined the thrombin potential in platelet rich plasma and in platelet poor plasma in 12 healthy volunteers and in 41 young stroke patients without medication, after two weeks use of aspirin (30 mg/day), and after two weeks use of fixed low-dose oral anticoagulants (phenprocoumon 0,75 mg/day). In the controls there was a mild but significant decrease (ca. 8%) of the thrombin potential in platelet rich plasma after two weeks on aspirin ($p=0.02$), whereas in the patients such an effect was seen only in the subgroup with platelet related hypercoagulability ($p<0.01$). In the patients with plasma based hypercoagulability the thrombin potential in the extrinsic pathway decreased after aspirin use ($p=0.005$). Platelet procoagulant activity decreased in our young stroke patients after aspirin treatment ($p=0.05$). Fixed low-dose phenprocoumon in the controls decreased the thrombin potential only in the extrinsic pathway ($p<0.05$), which effect was also seen in the patients ($p=0.03$). Also in the patient subgroup with platelet related hypercoagulability thrombin generation in platelet rich plasma decreased after phenprocoumon.

In conclusion: Aspirin modulates the hypercoagulable state as measured by the thrombin potential in young stroke patients, especially in those patients with platelet related hypercoagulability. However, it has only a mild effect on decreasing thrombin generation. We found no laboratory support for a potential higher effectiveness of fixed low-dose phenprocoumon compared to aspirin on the prevention of thromboembolic events.

Introduction

Secondary prevention is an important aspect in stroke management. Up till now, in most patients with ischaemic stroke or transient ischaemic attack (TIA) of presumed arterial origin antiplatelet therapy with aspirin is used. This therapy has limited, but clinically significant efficacy in the prevention of vascular events (stroke, myocardial infarction, or death from a vascular cause). In patients with a prior stroke or TIA antiplatelet therapy results in an absolute risk reduction of non-fatal stroke of 2% (8.2% in treated patients against 10.2% in controls).[10 54] There has been some discussion on the dose of aspirin to be used in secondary prevention of vascular events following TIA or stroke.[55 56] As no dose-effect relation has ever been demonstrated clinically, the dose in use depends on various factors. In the Netherlands most patients are treated with low-dose aspirin (30 mg), because a Dutch study showed no significant difference in efficacy of 30 mg or 283 mg of aspirin.[57]

Standard dose oral anticoagulation (with a prothrombin-time in International Standardized Ratio (INR) of 2-4) has only been proven beneficial in patients with cardioembolic stroke. In patients with TIA or minor stroke of presumed arterial origin however, standard dose oral anticoagulation carries a high risk of serious bleeding complications (mostly intracerebral haemorrhage). In a recent study comparing aspirin and standard dose oral anticoagulation in non-cardioembolic stroke, patients on anticoagulants had an odds of almost 20 of dying from bleeding complications, compared to those on aspirin.[11]

Low-dose anticoagulants might be an attractive alternative for patients with non-cardioembolic stroke. It seems likely that this would be associated with a lower bleeding risk than anticoagulants at standard intensity, and it might also require less frequent laboratory monitoring. Treatment with fixed low-dose warfarin (1 mg/day) reduced the incidence of deep vein thrombosis after major gynaecological surgery,[12 13] although the combination of aspirin and fixed low-dose warfarin had no additional effect over aspirin alone in patients after myocardial infarction.[15] Low-intensity warfarin (INR below 2.0) has no effect in the prevention of stroke in patients with non-rheumatic atrial fibrillation.[14 189 190] Obviously, the clinical efficacy of low-dose oral anticoagulation has not clearly been demonstrated.

Theoretically, patients with presumed platelet induced hypercoagulability would benefit from antiplatelet therapy, whereas a plasma based hypercoagulability would be better treated by damping the plasmatic coagulation system. As described in the previous chapters, the thrombin potential (ETP) offers the opportunity to distinguish between plasma or platelet based hypercoagulability.[150 151 160] The thrombin potential is a sensitive screening parameter for the coagulation system and for the

anticoagulant effect of medication. The ETP decreases to between 15 and 35% of normal by standard dose oral anticoagulation (INR 2.5-4.0) and by heparin administration.[160]

Neither fixed low-dose oral anticoagulants nor aspirin affects the standard clotting tests used to check antithrombotic treatment. Nevertheless, for effective prophylaxis an effect on the haemostatic function of the blood is likely to be required. It is conceivable that a decrease in the ETP, induced by aspirin or fixed low-dose oral anticoagulants, accompanies an effect in the prevention of thrombotic events in patients. Therefore, we investigated whether we could demonstrate a decrease in thrombin generation with the aid of the thrombin generation test. We determined ETP in platelet rich plasma and in platelet poor plasma in healthy volunteers and in young stroke patients without medication, after the use of aspirin, and after the use of fixed low-dose oral anticoagulants.

Subjects, materials and methods

Subjects

Twelve healthy volunteers (4 males, 8 females) participated in the study. Mean age was 32.8 (± 6.1) years. Four of the women used oral contraceptives. Five subjects had a first degree relative with vascular disease. Blood samples were taken after a period without medication, after two weeks use of 30 mg aspirin a day, and after at least two weeks use of 0.75 mg phenprocoumon a day.

There were 41 consecutive patients under age 50 with ischaemic stroke (three patients had a TIA). The data of these patients are described in detail in chapter 3.

Blood samples were taken after at least two weeks without aspirin, after two weeks use of 30 mg aspirin a day (most patients used aspirin since much longer), and after at least two weeks use of 0.75 mg phenprocoumon a day.

Materials

The materials used for the determination of the thrombin potential, platelet derived procoagulant activity and the thrombomodulin test are described in chapter 3 and in chapter 4.

Methods

The measurement of thrombin generation in platelet poor plasma and the thrombomodulin test are described in chapter 3. The determination of the

ETP in platelet rich plasma and the platelet derived procoagulant activity are described in detail in chapter 4.

INR, Factor II, VII and X

Prothrombin-time in INR was determined before medication, and after the use of aspirin and phenprocoumon in the twelve healthy volunteers. In the young stroke patients we determined prothrombin-time in INR without medication and after treatment with phenprocoumon. In the volunteers also Factor II, Factor VII and Factor X were determined without medication, and after the use of aspirin and phenprocoumon. Factor II, VII and X were measured using a factor assay procedure with Factor Deficient Plasma (Organon Teknika GmbH, Eppelheim, Germany).

Figure 5.1

To establish the relationship between ETP and INR we collected plasma from patients treated with oral anticoagulation at the Maastricht Thrombosis Service (head Dr. H.L.L. Frank). Plasma pools of 10 ml were made, each from 1 ml of individual plasma, selected to fall in a defined 0.1 INR interval. In this way 72 pools were obtained over the range of 0.9-3.2 INR. Of each of these pools the INR and the extrinsic ETP were determined. Pools were used to compensate for individual variation in clotting factors.

Statistical evaluation

Results are presented as median and 25 and 75 percentile. We used the Mann-Whitney test to analyze differences in distribution of continuous variables between groups and the Wilcoxon test to analyze paired samples. To analyze whether the relation between ETP in PRP and medication is mediated by platelet derived procoagulant activity, we used a linear regression model. The patient group was divided into three subgroups as described in chapter 3 and chapter 4 (fig. 4.1): patients with an ETP in PPP above the 66th percentile were considered to have an elevation of the ETP in PPP (group 2; n=14). Of the remaining 27 patients those with an ETP in PRP above the 66th percentile were considered to have an elevation of the ETP in PRP (group 3; n=9). The remaining patients were considered to have no clear abnormalities in coagulation as measured by the ETP (group1; n=18).

Results

Median values of the measurements in the controls without medication, after aspirin, and after phenprocoumon are listed in table 5.1. In the controls there was a mild but significant decrease (ca. 8%) of ETP in PRP after two weeks on aspirin (p=0.02), but not after two weeks on phenprocoumon. ETP in PPP

only decreased in the extrinsic system after two weeks on phenprocoumon (p=0.05). ETP in PPP did not change significantly after aspirin use. There was no significant change after use of medication in lag-phase, in platelet derived procoagulant activity or in percentage inhibition after addition of thrombomodulin. Prothrombin-time ratio did not change significantly neither after the use of aspirin nor after the use of phenprocoumon. Only factor X decreased significantly after the use of phenprocoumon (p=0.03) (table 5.1). Platelet derived procoagulant activity was significantly associated with ETP in PRP (regression coefficient 1.35; 95% confidence intervals 0.04-2.66). Median values of the measurements in 41 young stroke patients without medication, after aspirin, and after phenprocoumon are shown in table 5.2. ETP in PRP in our young stroke patients did not change significantly with aspirin, nor with phenprocoumon. ETP in the extrinsic coagulation system decreased significantly, both with aspirin and phenprocoumon (p=0.005 and p=0.03, respectively). In the intrinsic system no changes were observed after medication. Platelet derived procoagulant activity decreased after administration of aspirin (p=0.05), but not after phenprocoumon. The median of the INR in the patients was 0.95 (0.92-1.00) before treatment with phenprocoumon, and 0.97 (0.92-1.04) after treatment. There was no significant difference in INR before and after treatment with low fixed-dose oral anticoagulants.

Table 5.1. Median values (25-75 percentile) of the measurements without medication, after aspirin use, and after phenprocoumon use in twelve healthy volunteers.

	Without medication	Aspirin	Phenprocoumon
ETP in PRP	92 (85-107)	84 (75- 89)*	96 (82-101)
ETP extrinsic	108 (100-120)	108 (97-115)	105 (100-112)*
ETP intrinsic	99 (92-113)	100 (92-109)	99 (90-107)
PPA	73 (56-104)	78 (61-104)	78 (66- 91)
TM	43 (39- 49)	46 (38- 48)	43 (40- 48)
FII	103 (98-117)	103 (97-109)	102 (98-106)
FVII	114 (88-136)	115 (88-124)	107 (86-120)
FX	110 (97-128)	104 (99-126)	102 (94-120)*
INR	0.97 (0.94-1.08)	1.00 (0.95-1.06)	0.99 (0.94-1.08)

* $p < 0.05$

ETP: endogenous thrombin potential, PRP: platelet rich plasma, PPA: platelet derived procoagulant activity, TM: percentage inhibition of the ETP after addition of thrombomodulin, FII: prothrombin, FVII: factor VII, FX: factor X, INR: prothrombin-time in International Standardized Ratio.

Table 5.2. Median values (25-75 percentile) of ETP in PRP and PPP (extrinsic and intrinsic system) and platelet derived procoagulant activity (PPA) in young stroke patients without medication, after aspirin, and after phenprocoumon.

	Without medication	Aspirin	Phenprocoumon
ETP in PRP	111 (103-118)	108 (97-114)	107 (101-112)
ETP extrinsic	106 (93-121)	103 (91-114)*	101 (88-113)**
ETP intrinsic	105 (87-122)	102 (90-119)	100 (86-118)
PPA	90 (69-106)	73 (59- 98)***	85 (69-104)

* $p < 0.01$
** $p < 0.05$
*** $p = 0.05$

The results of the three patient subgroups are listed in table 5.3. In the patients with no elevation in ETP in PRP or PPP (group 1) no influence of medication was found. In the patients with an elevation of ETP in PPP (group 2), aspirin significantly reduced ETP in the extrinsic coagulation system. There was no influence of phenprocoumon on the ETP in PPP, neither in the extrinsic, nor in the intrinsic coagulation system. In the group with platelet related hypercoagulability, i.e. the group with an elevation of ETP in PRP only (group 3) both aspirin and phenprocoumon significantly reduced ETP in PRP (10% and 11%, respectively). Platelet derived procoagulant activity decreased also, but not significantly.

Figure 5.1 shows the relation between INR and ETP as obtained in 100 plasma pools at different INR levels.

Discussion

Aspirin

In the healthy volunteers we found a decrease of thrombin generation in platelet rich plasma as measured by the ETP after two weeks administration of aspirin, whereas in our young stroke patients we found no significant influence of aspirin on thrombin generation as measured by the ETP in PRP or PPP. However, within the patient group there were remarkable differences in influence of aspirin on thrombin generation. Based on whether the ETP was elevated or not, we distinguished three patients groups: group 1 with no abnormalities in thrombin generation, group 2 with an elevation of ETP in PPP, and group 3 with an elevation of ETP in PRP (fig. 4.1). Patients with platelet related hypercoagulability (group 3) had a decrease of ETP after aspirin use, although ETP did not return to normal values. Others also found a decrease of thrombin generation with aspirin.[152] [191] In another study high

Table 5.3. Median values (25-75 percentile) of ETP and platelet derived procoagulant activity (PPA) in the different subgroups of young stroke patients, without medication, after aspirin, and after phenprocoumon.

Group 1. Patients in whom no abnormalities in coagulation could be detected (n=18)

	Without medication		Aspirin		Phenprocoumon	
ETP in PRP	111	(94-113)	108	(94-113)	107	(95-113)
ETP extrinsic	96	(88-108)	96	(83-106)	94	(82-104)
ETP intrinsic	98	(78-105)	97	(86-103)	98	(81-105)
PPA	81	(64- 99)	71	(62-101)	85	(59- 92)

Group 2. Patients with a probable abnormality of the plasmatic coagulation system (n=14)

	Without medication		Aspirin		Phenprocoumon	
ETP in PRP	107	(101-111)	107	(96-114)	109	(99-115)
ETP extrinsic	122	(121-127)	115	(110-129)*	116	(112-130)
ETP intrinsic	128	(121-145)	124	(112-138)	129	(110-156)
PPA	86	(68- 91)	69	(59-101)	71	(63-101)

Group 3. Patients with platelet related hypercoagulability (n=9)

	Without medication		Aspirin		Phenprocoumon	
ETP in PRP	124	(119-134)	111	(94-115)**	110	(105-113)**
ETP extrinsic	97	(91-105)	96	(87-102)	93	(83- 99)
ETP intrinsic	92	(83-104)	94	(78-110)	93	(79- 98)
PPA	107	(78-117)	89	(53- 94)	96	(83-113)

* $p < 0.05$
** $p < 0.01$

concentrations of aspirin had no influence on thrombin peak concentration, but did prolong the lag-phase.[192] Thus, aspirin has a modest influence on thrombin generation in PRP, both in controls and in subgroups of patients.

There is a marginal effect of aspirin on thrombin generation in PPP in the patient group. In the patient subgroups the same effect of aspirin is only seen in those patients with a plasma based hypercoagulability. The influence of aspirin on the thrombin generation in PPP may be explained by the small anti-vitamin K effect that accompanies aspirin intake.[193-197] As aspirin is only a weak vitamin K antagonist, it was thought to bring about this effect only at very high dosages. Our results indicate that intake of a small dose over a longer period of time (most of our patients used aspirin for months, or even years) may still have this effect. Others also described that chronic treatment with aspirin reduced the total amount of thrombin formed.[191] The definite

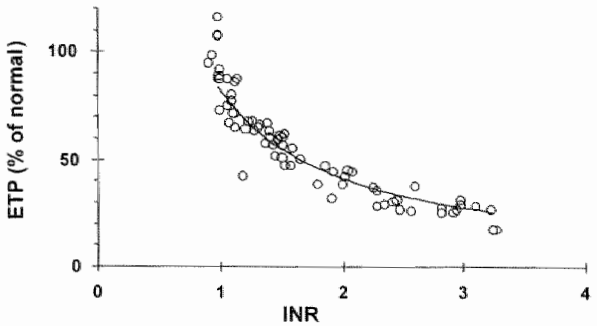


Figure 5.1. INR and ETP in treatment with oral anticoagulants (Figure by courtesy of H.C. Hemker, dep. of Biochemistry, University of Maastricht, The Netherlands).

proof that aspirin acts on thrombin generation in PPP is still lacking. We did not determine individual clotting factors in our patients. In the volunteers, where we did determine individual clotting factors, no effect of aspirin on thrombin generation in PPP was observed.

The effect of aspirin in PRP is probably related to a decrease in platelet procoagulant activity. Effects of aspirin in the presence of platelets have been observed before.[152 191 192] The mechanism by which aspirin inhibits the platelet contribution to thrombin generation remains unknown. It seems likely that inhibition of platelet-derived tromboxane formation is the principal antithrombotic mechanism of aspirin. However, tromboxane induced platelet activation is not involved in the exposure of platelet procoagulant phospholipids,[99 198] nor in thrombin induced platelet activation,[186] that occurs under our experimental circumstances. Thus, inhibition of the cyclooxygenase pathway might explain a prolongation of the lag-phase,[192] but not of the reduced amount of thrombin formed.[152 191] Other explanations that have been suggested are speculative and sometimes unlikely (acetylation of prothrombin or of GTP-binding proteins,[191] interaction with platelet membrane phospholipids[192]). It remains interesting to see that the aspirin effect is particularly obvious in those patients in which the thrombin generation in PRP is elevated. The mechanism by which this elevation is influenced is not elucidated, and further studies are required.

The decrease in thrombin generation (about 10%) that we observed with 30 mg of aspirin daily in our controls and in subgroups of patients is similar to those 1 hour after intake of 1 g of aspirin,[152] which shows that this aspirin effect is dose-independent and limited. This raises the interesting question of how much the ETP has to decrease to obtain adequate prophylaxis.

Oral anticoagulation

In the healthy volunteers we found a decrease in ETP in the extrinsic coagulation system of about 3%. The decrease in the intrinsic ETP was not statistically significant. Fixed low-dose anticoagulants reduced ETP in PRP with 11% only in the subgroup of patients with platelet related hypercoagulability. Because in this subgroup the same decrease in ETP (10%) was observed after aspirin use, fixed low-dose oral anticoagulants do not seem to have a additional effect over aspirin. Moreover, platelet derived procoagulant activity only decreased with aspirin, suggesting that aspirin could even be more effective than phenprocoumon.

'Mini-intensity' anticoagulation with warfarin has been reported to affect hypercoagulation markers, specifically F_{1+2} levels.[13 58 199] Fixed low-dose oral anticoagulants were effective in reducing the incidence of deep vein thrombosis after major gynaecological surgery[12 13] and in the prevention of thrombotic events in cancer patients,[200] but the combination of aspirin and fixed low-dose warfarin had no additional effect over aspirin alone in patients

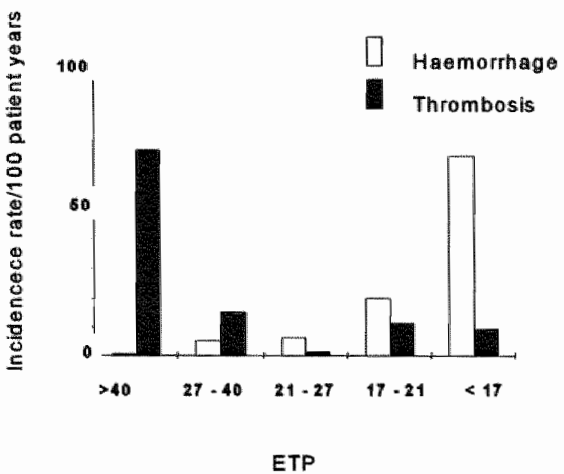


Figure 5.2. Thrombotic and haemorrhagic events during treatment with oral anticoagulants, as function of the percentage of the ETP (figure modified from Azar,[202] by courtesy of the author).

after myocardial infarction.[15] Low-intensity warfarin (INR below 2.0) had no effect in the prevention of stroke in patients with non-rheumatic atrial fibrillation.[14 189 190] This concurs with our findings, that provide no laboratory support for an important effect of fixed low-dose oral anticoagulants, neither in our young stroke patients, nor in healthy controls.

Neither in the controls nor in the young stroke patients did the use of fixed low-dose phenprocoumon influence the prothrombin-time expressed in INR. This finding is in accordance with a previous study, where INR did not change after six weeks of fixed low-dose warfarin, but only after adjusted dose warfarin.[200]

The effect of phenprocoumon in PPP is, on the whole, reflected in PRP, but not quantitatively. Theoretically, the two do not have to be identical. Apart from the procoagulant factors II, VII, IX and X, also the down-regulators protein C and protein S are vitamin K dependant. The equilibrium between pro- and anticoagulant effects is determined by the types of phospholipids available.[201] In PPP phospholipids are added, but in PRP they are provided by the platelets, so the quantitative effects of anticoagulation need not be identical.

The minimal effect on thrombin generation obtained by 0.75 mg phenprocoumon per day raises two questions:

1. What is the minimal anticoagulant effect that might give safe protection against thromboembolic events in young stroke patients?
2. How could this effect be obtained and is there a need for laboratory control?

Ad 1. The risk of myocardial reinfarction as a function of the INR are known from previous studies.[202 203] We determined the relation between INR and ETP (fig. 5.1) and modified the data of Azar in terms of inhibition of the ETP (fig. 5.2). This allows us to conclude that 60% inhibition of the ETP suffices to reduce the risk of myocardial infarction, whereas bleeding complications increase if the inhibition exceeds 80%. Unluckily, cerebral bleeding in the diseased brain occurs more readily than bleeding in general.[11]

If the efficacy of aspirin would be due to the decrease of the ETP in PRP, than 10% decrease of the ETP in PRP has a moderate but definite effect in the secondary prevention of thromboembolic events, and it would be a reasonable guess to aim at the range of 20-40% of inhibition of the ETP as the window for secondary prevention of cerebral infarction.

Ad 2. From figure 5.1 it can be seen that 20-40% inhibition of the ETP corresponds to a range of INR between 1.1 and 1.4. It is also evident from the hyperbolic relationship between INR and ETP that the INR is extremely insensitive in this range, and hence unsuitable as a means of laboratory control.

The question how to reach a 20-40% of inhibition of the ETP with a fixed dose of a vitamin K antagonist can not be answered at this moment. From the

data available at the Maastricht Thrombosis Service we know that a stable anticoagulation at an INR 2.5-4.2 (inhibition of ETP 60-70%), requires 3 ± 0.6 mg of phenprocoumon. This means that with a standard dose of 3 mg phenprocoumon 15% of a population would be anticoagulated at a < 60% level and 15% at a >70% level. This suggests that the variation in interindividual responses is too large to obtain stable anticoagulation in the relatively narrow range of 20-40% inhibition with a fixed dose of phenprocoumon.

The effect of aspirin in the secondary prevention of stroke is clinically significant, but still moderate. Antiplatelet therapy results in an absolute risk reduction in non-fatal stroke of 2% (8.2% in treated patients, 10.2% in controls) in patients with a prior stroke or transient ischaemic attack (TIA).[10 54] This clearly suggests the need for more effective antithrombotic therapy. In our young stroke patients with platelet related hypercoagulability both aspirin and fixed low-dose phenprocoumon resulted in a significant reduction of 10% of ETP in PRP, but the values of ETP still remained elevated. In the other patient subgroups there was no significant influence of aspirin or phenprocoumon on ETP in PRP. Maybe in subgroups of patients other (antiplatelet) therapy would be more beneficial. In the search for those drugs the ETP could be a useful screening test to study the effect of medication on thrombin generation.
In conclusion: Aspirin modulated the hypercoagulable state as measured by the ETP in young stroke patients, especially in those patients with platelet related hypercoagulability. The effect on decreasing thrombin generation however, is only mild. There is no laboratory support for a more beneficial effect of fixed low-dose phenprocoumon compared to aspirin.

Chapter 

General discussion

When a young person develops an ischaemic stroke without a definite cause despite extensive investigations, it means that current knowledge is insufficient to identify the cause of that stroke. In up to 50% of young stroke patients no definite cause can be found.[2 16 18-22] Many disorders in various pathophysiological domains may contribute to the cause of the stroke, among which are rheologic or haemostatic abnormalities. These abnormalities may play a more important role in some ischaemic stroke subtypes than in others; e.g. a definite stroke cause is less often identified in lacunar than in territorial ischaemic stroke.[23] With the experiments described in this thesis we aimed to contribute to the understanding of pathogenetic mechanisms involving haemostatic function in young patients suffering ischaemic stroke. Knowledge of such mechanisms could foster a rational approach towards the prevention of recurrent vascular events in these patients.

Enhanced red blood cell aggregation can increase blood viscosity and in this way diminishes blood flow.[67 68 70-72] Several studies found enhanced red blood cell aggregation to be related to ischaemic stroke. Some ascribed such correlation to an increased fibrinogen level.[6 74] However, we found an enhanced red blood cell aggregation in young stroke patients not related to the fibrinogen level. Therefore, our findings indicate that some other factor, maybe some plasma protein other than fibrinogen, is responsible for the increased aggregability of red blood cells in these patients. Another possible explanation is that enhanced red blood cell aggregation is secondary to platelet activation,[77] because thrombospondin, one of the contents of the platelet α-granules, may induce erythrocyte aggregation,[81] whereas erythrocyte aggregation in turn can increase platelet procoagulant responses. Under certain conditions erythrocytes can develop procoagulant activity by a transbilayer movement of procoagulant phospholipids,[65] thereby influencing the haemostatic functions of the blood. Erythrocytes can enhance platelet activity,[79] whereas enhanced red blood cell aggregation can exacerbate platelet aggregation.[80] Further study on erythrocyte-platelet interactions may eventually allow a better understanding of the role of enhanced erythrocyte aggregation in the pathogenesis of ischaemic stroke. This will be a point of future interest.

The coagulation mechanism in plasma that is responsible for the generation and subsequent inhibition of thrombin in clotting blood may lead to a thrombotic tendency when producing too much free thrombin (hypercoagulability). The ability to detect coagulation abnormalities as a possible contributor to the development of stroke depends on the availability of a sensitive test of the coagulation system. Global clotting assays are often used to detect hypercoagulability. However, the clotting time represents only the 10 nM thrombin required to convert fibrinogen into fibrin, whereas

thrombin generation continues until a peak of about 200 nM is reached. Therefore, clotting times are much more sensitive to hypocoagulability than to hypercoagulability. The time integral of thrombin formation, i.e. the endogenous thrombin potential (ETP), is an overall indicator of the plasma coagulability, and can be used as a screening parameter of the coagulation system. Measurement of the ETP in platelet rich and in platelet poor plasma allows determination of hypercoagulability, whereas it also differentiates between abnormalities in the plasmatic and the cellular coagulation system. The possibility to separately identify patients with hypercoagulability either due to the plasmatic coagulation system or related to platelets may eventually allow a more rational therapeutic approach. We found a plasma based hypercoagulability in about one third of our young stroke patients, whereas in about a quarter of these patients a platelet related hypercoagulability could be identified. A high thrombin generation in platelet rich plasma possibly may increase the risk of stroke, whereas plasma based hypercoagulability may increase the risk of recurrent stroke. These findings indicate that the role of hypercoagulability in the development of cryptogenic stroke in the young may be far more important than so far assumed.[5 24 25] What specific coagulation abnormalities cause this hypercoagulability is not yet known, but our findings may direct towards further exploration of underlying causes. The ETP can thereby be helpful in identifying the type of hypercoagulability (plasma based or due to platelets). Moreover, with the ETP we were able to exclude hypercoagulability in about 50% of our patients. Therefore, using the ETP may limit the need for further extensive (and often expensive) laboratory investigations for specific coagulation abnormalities in these patients.
Measurement of thrombin formation in platelet rich plasma is valuable because it includes the influence of the blood platelets, which *in vivo* provide the phospholipid surface necessary for the prothrombinase anchorage. Therefore, the ETP in platelet rich plasma represents the situation *in vivo* better than thrombin generation in platelet poor plasma. However, other blood cells, the vessel wall, and flow characteristics which are present *in vivo*, do not play a role in the thrombin generation test. Nevertheless, the ETP in platelet rich plasma reveals hypercoagulability in young stroke patients that is not detected by other tests. In its present form however, it is not an easy test that can be applied on a routine basis. One test requires two working hours by experienced technicians. Therefore, it would be very advantageous to develop an automatised procedure for the thrombin generation test in platelet rich plasma. This already exists for the measurement of thrombin formation in platelet poor plasma.

We demonstrated a high platelet procoagulant activity and an elevated vWF in young stroke patients, and both were related to thrombin generation in platelet rich plasma. vWF is one of the necessary mediators in the mechanism that brings out platelet procoagulant activity in platelets. Thrombin induces

platelet procoagulant activity in a GPIIb/IIIa dependent process. Fibrin requires GPIb for making the platelets procoagulant. vWF is a necessary cofactor in both mechanisms.[7 104] Activated platelets provide the procoagulant surface needed for the activation of factor X and prothrombin.[91 101] The moment on which the platelets massively expose their procoagulant surface and shed procoagulant microvesicles, thrombin will massively be generated. Thrombin in turn, is one of the most potent platelet activators. The generation of thrombin and the development of platelet procoagulant activity are thus closely linked, which also can be deduced from the correlation between ETP and platelet procoagulant activity in our patients. The development of platelet procoagulant activity, as indicated above, is in more than one way dependent on vWF. We found an elevated concentration of vWF in young stroke patients, which was related to thrombin generation and platelet procoagulant activity. Obviously, vWF is not merely a marker of endothelial damage, but plays an essential role in coagulation through its effect on platelet activation. Our findings therefore provide a pathophysiological explanation for the epidemiological observation that relates an increased concentration of vWF to the occurrence of stroke[8 139 140] and to the increased mortality in stroke survivors.[9] Moreover, vWF could play a crucial role in the induction of a prethrombotic state in a subgroup of young stroke patients. Therefore, inhibition of vWF platelet receptors, like GPIIb/IIIa or GPIb, can influence the interaction of vWF with platelet activation and subsequent thrombin generation. Thrombin generation decreased after addition of GPIIb/IIIa antagonists, indicating that GPIIb/IIIa has a function in the platelet procoagulant response.[204]

In elderly stroke patients atherosclerosis is the most frequent cause of stroke. Coagulation enzyme activity increases with age,[205] and might be the basis of a thrombotic tendency. Therefore, a prethrombotic state may contribute to the development of stroke in elderly patients as well. A coexistent atherosclerosis could obscure the role of such a coagulopathy, but may on the other hand induce hypercoagulability. By using the ETP as a screening parameter for the coagulation system, the role of coagulation abnormalities in elderly stroke patients may be elucidated. Moreover, since stroke incidence in elderly patients is much higher, in such a study attention could be given to distinguish between stroke subtypes. Since the pathogenesis of lacunar and territorial infarcts is different,[155 206] it could be possible that there are differences in the prevalence of hypercoagulability between stroke subtypes. We were not able to demonstrate differences between lacunar and territorial stroke due to the small numbers in the different stroke subgroups. Insight into pathophysiological mechanisms of stroke subtypes may have important consequences for tailored therapeutical decisions. Recently, this was demonstrated by the excess of fatal bleedings in TIA or minor stroke patients with white matter lesions during oral

anticoagulant therapy,[11] indicating that consequences of antithrombotic therapy are different in different subsets of stroke patients.

Secondary prevention is an important aspect in stroke management. Up till now, most patients with stroke or TIA of presumed arterial origin are treated with antiplatelet therapy. This results in an absolute risk reduction in non-fatal stroke of 2% (8.2% in treated patients, 10.2% in controls).[10] Therefore, there is a need for a more potent modality, with a low risk of bleeding complications. Theoretically, patients with presumed platelet induced hypercoagulability would benefit from antiplatelet therapy, whereas any effect of fixed low-dose oral anticoagulants is more likely in patients with hypercoagulability due to the plasmatic coagulation system. A more rational prescription of a preventive drug requires recognition and classification of plasma or platelet based hypercoagulability. The ETP is sensitive screening parameter for the coagulation system and is known to be decreased by heparin, standard dose oral anticoagulants, and antiplatelet drugs (aspirin, GPIIb/IIIa blockers). The ETP allows identification of patient subgroups in which antiplatelet drugs, oral anticoagulants, or other newly developed drugs could be more rationally tested, instead of lumping all stroke patients together in therapeutic experiments. Furthermore, in the future the ETP might be useful as a screening test in (trials on) primary prevention to identify patients at high risk for thromboembolic events.[207-209]

Among the healthy volunteers who used aspirin we found a decrease in thrombin generation in platelet rich plasma of about 8%, while in young stroke patients thrombin generation in platelet rich plasma decreased by 10%, but only in the subgroup with platelet based hypercoagulability. Other authors also observed a decrease in thrombin generation by about 10%, possibly through its effect on the production of procoagulant phospholipids.[152 191 192] Indeed, platelet procoagulant activity in our young stroke patients decreased significantly after aspirin use, but not in the controls. Furthermore, aspirin also decreased the extrinsic ETP in the patient subgroup with plasma based hypercoagulability. Inhibition of platelet-derived tromboxane formation is the principal recognized antithrombotic mechanism of aspirin. However, also tromboxane-independent actions of aspirin might be involved in its antithrombotic effect, and underlie its influence on thrombin generation.[191] Aspirin can influence the plasma levels of the vitamin K-dependent coagulation factors, though this effect was only seen at high dosages (100 mg/kg by intraperitoneal injection; 1 g/kg by a single intramuscularly injection).[193-197] Apart from the dose, also duration of aspirin treatment could influence coagulation factors. Therefore, a decrease in thrombin generation might also have been induced by long-term low-dose aspirin treatment in our patients.

Low-dose aspirin treatment in our study decreased thrombin generation only in those patients with an elevated ETP. This finding could mean that in the secondary prevention of stroke some patients may require high-dose aspirin, a different antiplatelet drug,[210] or a combination of drugs, such as aspirin with dipyridamole, as recently found in the ESPS2.[211] Fixed low-dose phenprocoumon did not reduce thrombin generation more than aspirin did. The dose of phenprocoumon in our study population was 0,75 mg a day. Maybe a higher dose would have been more effective. At a higher dose, however, laboratory monitoring would be necessary, because there are patients who will become fully anticoagulated with 1.5 mg/day phenprocoumon. Low-dose oral anticoagulants were proven efficacious in the prevention of thrombotic complications in some studies in patients after major surgery.[12 13] Low-dose oral anticoagulants may be effective in some patients, but not in all and in our opinion some laboratory control will always be required.

Since the ETP is universally inhibited by all antithombotics, no matter what the antithrombotic mechanism is, it is very sensitive for the evaluation of the potential therapeutic effect of any therapeutic modality. It offers an attractive opportunity to evaluate the effect of drug treatment on thrombin generation, before starting a large randomized trial without differentiating what patients may and what patients likely will not benefit. Restricted entry of patients with a high probability of therapeutic effect would make any clinical trial on antithrombotics in many aspects much more feasible.

Various antithrombotic drugs are currently being evaluated. Thienopyridines, such as ticlopidine or clopidogrel, are valuable alternatives if platelet activation results from shear stress or ADP. Clopidogrel has been proven effective in reducing vascular events, though the absolute risk reduction compared to aspirin was only 0.5%.[210 212] GPIIb/IIIa antagonists are considerably more potent than aspirin in acute vessel obstructions,[213 214] but also have a substantially higher risk of bleeding.[198 215 216] It seems likely that the best target for new antithrombotic drugs is the inhibition of thrombin generation. To coin such drugs the ETP may be the screening procedure of choice. Therefore, making the ETP in platelet rich plasma available for routine use would be most welcome and could facilitate further basic as well as clinical research.

Chapter 

Summary

Effective prevention of stroke is best applied on basis of the pathophysiological mechanism underlying the stroke. In many young stroke patients however, this mechanism remains uncertain. Conflicting data exist about hypercoagulability as a possible contributor to ischaemic stroke in young patients. Extensive laboratory screening for prethrombotic states in stroke patients is limited to the recognition of a number of known diseases that cause hypercoagulability, such as the factor V Leiden mutation, deficiencies of proteins C and S, hyperhomocysteinaemia etc. Such screening is expensive and not widely available. Besides, it often provides only negative information, because only specific, known causes of hypercoagulability can be investigated. Instead of investigating a large number of possible causes of hypercoagulability, it would be a great advantage to have a screening parameter of the coagulation system as a whole, i.e. the plasmatic coagulation system together with the platelets. In this way further specific testing could be limited to patients with an identified hypercoagulability. It was the main aim of this thesis to investigate whether an overall test, the endogenous thrombin potential (ETP), can be used as such a screening parameter in young stroke patients. With the ETP, measured both in platelet rich plasma and in platelet poor plasma, it is possible to differentiate between hypercoagulability due to platelets, or hypercoagulability due to the plasmatic coagulation system. Also the influence of medication on the hypercoagulability can be studied with the ETP. Apart from cardioembolic stroke, secondary stroke prevention up till now is limited to antiplatelet therapy, which is only moderately effective. In specific subgroups other medication, like fixed low-dose oral anticoagulants, may be more effective in preventing vascular events. With the use of the ETP it may be possible to stratify young stroke patients in groups that will benefit from either oral anticoagulant or antiplatelet therapy.

In *Chapter 2* we set out to explore the role of erythrocyte aggregation in young patients with non-cardioembolic stroke. Enhanced red blood cell aggregation is considered as a factor related to the pathogenesis of stroke in elderly patients, in whom enhanced red blood cell aggregation is correlated with increased fibrinogen. In young stroke patients we found an enhanced red blood cell aggregation compared to young controls, both in the early and in the late phases, whereas fibrinogen was normal. Red blood cell aggregation was significantly associated with stroke after adjusting for differences in fibrinogen, hematocrit and erythrocyte sedimentation rate. Red blood cell aggregation was higher in elderly patients than in elderly controls. In elderly patients increased fibrinogen was associated with stroke. We concluded that enhanced red blood cell aggregation independently relates to stroke in young people, which may suggest that enhanced red blood cell

aggregation contributes to stroke cause, whereas in elderly patients any such effect is probably explained by confounding by raised fibrinogen.

In *Chapter 3* we determined whether young stroke patients have abnormalities in the plasmatic coagulation system. Furthermore, to investigate whether there were any disturbances in the protein C pathway, we added an activator of protein C, thrombomodulin, to the thrombin generation assay. With the use of the ETP in platelet poor plasma as a screening parameter of the plasmatic coagulation system, we found a plasma based hypercoagulability in about one third of young stroke patients. Patients with recurrent stroke had a significantly higher ETP than those without. After the addition of thrombomodulin five patients had an insufficient inhibition of thrombin generation, indicating an abnormality in the protein C pathway. Abnormalities in the protein C pathway are easily detectable with the thrombomodulin test, and may contribute to the development of stroke in the presence of other vascular risk factors. In patients with an identified hyperactive coagulation system, further investigations into the cause of this plasma based thrombotic tendency are warranted.

In *Chapter 4* we tested whether young stroke patients have a hypercoagulability related to the blood platelets. Comparing the ETP in platelet rich plasma to the ETP in platelet poor plasma allows distinction between platelet related or plasma based hypercoagulability. The von Willebrand factor is a necessary mediator in the mechanism that brings out procoagulant activity in platelets. Therefore, we also determined platelet procoagulant activity and the concentration of von Willebrand factor. The ETP in platelet rich plasma was significantly higher in young stroke patients than in controls. Platelet procoagulant activity and von Willebrand factor were also significantly higher in patients than in controls. High ETP was significantly associated with stroke. In a linear regression model von Willebrand factor concentration was associated with platelet procoagulant activity and with the ETP, whereas platelet procoagulant activity also was associated with the ETP. We concluded that thrombin generation in platelet rich plasma is elevated in young stroke patients. Both platelet derived procoagulant activity and von Willebrand factor are increased in young stroke patients and are related to the ETP in platelet rich plasma. This indicates that among the numerous causes that may be behind an increased ETP, a high concentration of von Willebrand factor is of significant importance. Our findings provide a pathophysiological explanation for the epidemiological observation that relates increased concentration of von Willebrand factor to the occurrence of stroke.

In *Chapter 5* we investigated the influence of aspirin and of fixed low-dose oral anticoagulants on thrombin generation in healthy volunteers and in young stroke patients. It would be advantageous if patients with hypercoagulability, either due to the plasmatic coagulation system or related to platelets, could be separately identified, as this may eventually allow a more rational prescription of a preventive drug. It is conceivable that a decrease in the thrombin generation, induced by aspirin or fixed low-dose oral anticoagulants, lowers the risk of thrombotic events in patients. In the controls there was a mild but significant decrease (ca. 8%) of the ETP in platelet rich plasma after two weeks of aspirin intake (30 mg/day), whereas in the patients such an effect was seen only in the subgroup with platelet related hypercoagulability. In the patients with plasma based hypercoagulability the ETP in the extrinsic pathway decreased after aspirin use. Platelet procoagulant activity decreased in our young stroke patients after aspirin treatment. Fixed low-dose phenprocoumon (0,75 mg/day) in the controls decreased only the extrinsic ETP in platelet poor plasma, which effect was also seen in the patients. Also in the patient subgroup with platelet related hypercoagulability thrombin generation in platelet rich plasma decreased after phenprocoumon. We concluded that aspirin modulated the hypercoagulable state as measured by the ETP in young stroke patients, especially in those patients with platelet related hypercoagulability. However, it has only a mild effect on decreasing thrombin generation. We found no laboratory support for a potential higher effectiveness of fixed low-dose phenprocoumon compared to aspirin on the prevention of thromboembolic events.

In *Chapter 6* we discussed the results of our studies on enhanced erythrocyte aggregation and on hypercoagulability in young stroke patients, in relationship with current and potential future therapeutical options for secondary stroke prevention. We recommend further study on ETP detectable hypercoagulability in elderly stroke patients and in patient subgroups. The availability of a routinely usable ETP measurement in platelet rich plasma would be of great advantage to such further studies. It would also offer an attractive opportunity to tailor future therapeutic modalities to subgroups of patients, identified with the ETP.

Chapter 

Samenvatting

Preventie van het herseninfarct is het meest effectief indien dit gericht kan worden op de onderliggende oorzaak van dit herseninfarct. Bij veel jonge patiënten met een herseninfarct wordt, ondanks uitgebreid aanvullend onderzoek, echter geen duidelijke oorzaak aangetoond. In de literatuur worden verschillende getallen genoemd omtrent hypercoagulabiliteit als mogelijke factor die bijdraagt aan het ontstaan van een herseninfarct op jonge leeftijd. Uitgebreid laboratorium onderzoek naar een zogenaamde 'prethrombotic state' bij deze patiënten is beperkt tot de herkenning van een aantal bekende aandoeningen die hypercoagulabiliteit veroorzaken, zoals factor V Leiden mutatie, deficiëntie van proteïne C of S, hyperhomocysteinemie, enz. Dergelijk screenend onderzoek is duur, en niet overal uitvoerbaar. Daarnaast levert dit vaak slechts negatieve resultaten op, omdat alleen specifieke, al bekende oorzaken van hypercoagulabiliteit kunnen worden onderzocht. Het zou een groot voordeel zijn als men zou beschikken over een screeningsparameter voor het totale stollingsysteem (dus het plasmatisch stollingsysteem samen met de bloedplaatjes). Dit zou als voordeel hebben dat verder onderzoek naar stollingsafwijkingen beperkt zou kunnen worden tot die patiënten met een geïdentificeerde hypercoagulabiliteit. Het voornaamste doel van dit proefschrift was dan ook om te onderzoeken of zo'n test gebruikt kan worden als een dergelijke screeningsparameter bij jonge patiënten met een herseninfarct. De 'endogene trombine potentiaal' (ETP) is een dergelijke test, waarmee een totale indruk kan worden verkregen over het stollingssysteem. Met behulp van de ETP, gemeten in plaatjesrijk en in plaatjesarm plasma, is het bovendien mogelijk onderscheid te maken tussen hypercoagulabiliteit ten gevolge van de bloedplaatjes, en hypercoagulabiliteit ten gevolge van het plasmatisch stollingsysteem. Ook kan het effect van medicatie op de hypercoagulabiliteit met behulp van de ETP worden bestudeerd. Met uitzondering van het herseninfarct ten gevolge van een cardiale embolie, is de secundaire preventie van het ischemisch CVA tot op heden beperkt tot plaatjes-aggregatie remmers; een therapie die slechts een beperkte effectiviteit heeft. In specifieke subgroepen zou andere medicatie, zoals bijvoorbeeld een vaste, lage dosis orale anticoagulantia, wellicht meer effectief zijn. Met behulp van de ETP is het mogelijk bepaalde subgroepen patiënten te onderscheiden, die wellicht meer baat hebben bij orale anticoagulantia, danwel aspirine.

In *Hoofdstuk 2* is de rol onderzocht van aggregatie van rode bloedcellen bij jonge patiënten met een herseninfarct. Een verhoogde erytrocyten aggregatie wordt beschouwd als een factor die gerelateerd is aan de pathogenese van het herseninfarct bij oudere patiënten. Bij deze patiënten is de verhoogde erytrocyten aggregatie gecorreleerd aan een verhoogd fibrinogeen. Bij jonge patiënten met een herseninfarct vonden wij een

verhoogde erytrocyten aggregatie vergeleken met controlepersonen, zowel in de acute als in de late fase na het infarct, terwijl de fibrinogeen concentratie normaal was. Erytrocyten aggregatie was significant geassocieerd met het herseninfarct na correctie voor verschillen in fibrinogeen, hematocriet en bezinking. Ook bij de oudere patiënten was de erytrocyten aggregatie verhoogd, vergeleken met oudere controlepersonen. Bij deze oudere patiënten bleek een verhoogd fibrinogeen geassocieerd te zijn met het optreden van een herseninfarct. Wij concludeerden dat een verhoogde erytrocyten aggregatie onafhankelijk gerelateerd is aan het optreden van een herseninfarct op jonge leeftijd, hetgeen erop zou kunnen wijzen dat een verhoogde erytrocyten aggregatie bijdraagt aan de oorzaak van dit herseninfarct. Bij oudere patiënten lijkt een verhoogde erytrocyten aggregatie voornamelijk te worden veroorzaakt door een verhoogd fibrinogeen.

In *Hoofdstuk 3* bepaalden we in hoeverre jonge patiënten met een herseninfarct afwijkingen hebben in het plasmatisch stollingssysteem. Daarnaast onderzochten wij ook of er afwijkingen bestonden in de 'protein C pathway', door een activator van proteïne C (thrombomoduline) toe te voegen aan de trombine generatie test. Met behulp van de ETP in plaatjesarm plasma, vonden we een plasma-gebonden hypercoagulabiliteit bij ongeveer één derde van de jonge patiënten met een herseninfarct. Patiënten die een recidief herseninfarct hadden doorgemaakt, bleken een significant hogere ETP te hebben dan de overige patiënten. Na toevoegen van thrombomoduline hadden vijf patiënten onvoldoende remming van de trombine generatie, hetgeen wijst op een onvoldoende functie van de 'protein C pathway'. Afwijkingen in de protein C pathway zijn gemakkelijk op te sporen met de thrombomoduline test, en kunnen bijdragen aan het ontstaan van een herseninfarct, vooral indien er ook nog andere vasculaire risicofactoren aanwezig zijn. Bij patiënten met een aangetoonde afwijking in het plasmatisch stollingsysteem is verder onderzoek naar de oorzaak van deze hypercoagulabiliteit aangewezen.

In *Hoofdstuk 4* onderzochten we of jonge patiënten met een herseninfarct hypercoagulabiliteit hebben, gerelateerd aan de bloedplaatjes. Door de ETP in plaatjesrijk plasma te vergelijken met de ETP in plaatjesarm plasma, kan onderscheid worden gemaakt tussen hypercoagulabiliteit op basis van het plasmatisch stollingssysteem, danwel de bloedplaatjes. De von Willebrand factor is een noodzakelijke mediator bij het mechanisme dat procoagulante activiteit in plaatjes teweeg brengt. Daarom onderzochten we ook de plaatjes procoagulante activiteit en de von Willebrand factor concentratie. De ETP in plaatjesrijk plasma was significant hoger bij de patiënten in vergelijking met controlepersonen. Ook de plaatjes procoagulante activiteit en de von Willebrand factor waren significant hoger in patiënten dan in de controles.

Een hoge ETP was significant geassocieerd met een herseninfarct. In een lineair regressiemodel was de von Willebrand factor geassocieerd met de plaatjes procoagulante activiteit en met de ETP, terwijl de plaatjes procoagulante activiteit ook geassocieerd was met de ETP. Wij concludeerden dat de trombine generatie is verhoogd bij jonge patiënten met een herseninfarct. Zowel de plaatjes procoagulante activiteit als de von Willebrand factor zijn verhoogd bij jonge patiënten met een herseninfarct, en zij zijn gerelateerd aan de ETP in plaatjesrijk plasma. Dit wijst erop dat onder de verschillende oorzaken die ten grondslag kunnen liggen aan een verhoogde ETP, een hoge concentratie van de von Willebrand factor een belangrijke rol speelt. Deze bevindingen geven een pathofysiologische verklaring voor de epidemiologische observatie dat een verhoogde von Willebrand factor concentratie is gerelateerd aan het optreden van een herseninfarct.

In *Hoofdstuk 5* hebben we de invloed van aspirine en van een vaste, lage dosering orale anticoagulantia op de trombine generatie onderzocht, zowel bij jonge patiënten met een herseninfarct als bij gezonde vrijwilligers. Het is aannemelijk dat een vermindering van de trombine generatie, door behandeling met aspirine of orale anticoagulantia, samengaat met een vermindering van het risico op een trombo-embolische gebeurtenis bij patiënten. Bij de controlepersonen bestond een verlaging van de ETP (ca. 8%) in plaatjesrijk plasma na aspirine gebruik (30 mg/dag) gedurende twee weken, terwijl bij de patiënten een dergelijk effect alleen werd gezien bij de subgroep met plaatjes-gerelateerde hypercoagulabiliteit. Bij patiënten met een plasma-gebaseerde hypercoagulabiliteit daalde de extrinsieke ETP tijdens aspirine gebruik. De plaatjes procoagulante activiteit daalde bij onze patiënten tijdens het gebruik van aspirine. Een vaste, lage dosering phenprocoumon (0,75 mg/dag) bij de controlepersonen verminderde alleen de extrinsieke ETP. Hetzelfde effect werd bij de patiënten gezien. Bij de subgroep patiënten met een plaatjes-gerelateerde hypercoagulabiliteit daalde ook de ETP in plaatjesrijk plasma na gebruik van phenprocoumon. We concludeerden dat aspirine de hypercoagulabiliteit, gemeten met behulp van de ETP, bij jonge patiënten met een herseninfarct beïnvloedt, speciaal in die groep met een plaatjes-gerelateerde hypercoagulabiliteit. Het effect op de trombine generatie is echter slechts mild. Wij vonden geen laboratorium ondersteuning voor het idee dat een vaste, lage dosering phenprocoumon effectiever is dan aspirine bij de preventie van tromboembolische gebeurtenissen.

In *Hoofdstuk 6* hebben we de resultaten van ons onderzoek naar een verhoogde erytrocyten aggregatie en naar hypercoagulabilitieit bij jonge patiënten met een herseninfarct besproken. Ook bediscussieerden we onze

bevindingen in relatie tot huidige en toekomstige therapeutische opties voor de secundaire preventie van het herseninfarct. Wij adviseren verder onderzoek naar hypercoagulabiliteit, onderzocht met behulp van de ETP, bij oudere patiënten en bij subgroepen patiënten. Het beschikbaar zijn van een geautomatiseerde versie van de ETP in plaatjesrijk plasma zou bij dergelijk studies van groot nut zijn. Dit zou ook de mogelijkheid bieden om toekomstige therapeutische alternatieven te richten op subgroepen van patiënten, die met behulp van de ETP te onderscheiden zijn.

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Curriculum vitae

Karin Faber werd geboren op 6 november 1965. In 1984 deed ze eindexamen VWO aan het Willem Lodewijk Gymnasium te Groningen. Hierna studeerde ze Geneeskunde aan de Rijksuniversiteit Groningen. In 1992 behaalde ze haar arts-examen, waarna ze direct is begonnen als assistent Neurologie in het Medisch Spectrum Twente, Enschede. Hier werd een aanvang gemaakt met het onderzoek dat uiteindelijk tot dit proefschrift heeft geleid. In 1993 kwam ze, aanvankelijk als AGNIO en later in opleiding, in dienst van het Academisch Ziekenhuis Maastricht (opleider Prof.dr. J. Troost). In Maastricht werd het onderzoek voortgezet onder leiding van Prof.dr. H.C. Hemker en Prof.dr. J. Troost. De opleiding Neurologie zal medio 2000 zijn afgerond.